ΠΑΝΕΠΙΣΤΗΜΙΟ ΔΥΤΙΚΗΣ ΑΤΤΙΚΗΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΤΡΟΦΙΜΩΝ

ΤΜΗΜΑ ΕΠΙΣΤΗΜΩΝ ΟΙΝΟΥ ΑΜΠΕΛΟΥ ΚΑΙ ΠΟΤΩΝ

Διδακτορική Διατριβή
του Ντουρτόγλου Γεώργιου (ΑΜ 19301)

«Μελέτη της επίδρασης του παλλόμενου ηλεκτρικού πεδίου στην παραγωγή ποτών»

Συμβουλευτική Επιτροπή:

1. **Ταταρίδης Π., Επίκουρος Καθηγητής, Πανεπιστήμιο Δυτικής Αττικής (επιβλέπων)**
2. **Χατζηλαζάρου Α., Καθηγήτρια Πανεπιστήμιο Δυτικής Αττικής**
3. **Λαλάς Σ., Καθηγητής, Πανεπιστήμιο Θεσσαλίας**

UNIVERSITY OF WEST ATTICA

SCHOOL OF FOOD SCIENCE

DEPARTMENT OF WINE, VINE & BEVERAGE SCIENCES

Doctoral Thesis
of George Dourtoglou (AM 19301)

«Study of the effect of the pulsating electric field on the production of beverages»

Advisory Committee:

1. **Tataridis P., Assistant Professor, University of West Attica (supervisor)**
2. **Chatzilazarou A., Professor, University of West Attica**
3. **Lalas S., Professor, University of Thessaly**

"Εάν έχω δει πιο μακριά από τους άλλους, είναι γιατί στεκόμουν στους ώμους γιγάντων." - Ισαάκ Νεύτων

Στην Ελπίδα

Ευχαριστώ βαθιά τους ανθρώπους που με συντροφεύσαν σε αυτό το ταξίδι.

Μέλη Τριμελούς Συμβουλευτικής Επιτροπής

Α/Α	ΟΝΟΜΑ ΕΠΩΝΥΜΟ	ΥΠΟΓΡΑΦΗ
1	**Ταταρίδης Παναγιώτης**	Panagiotis Tataridis Digitally signed by Panagiotis Tataridis Date: 2023.09.08 11:30:41 +03'00'
2	**Χατζηλαζάρου Αρχοντούλα**	Archontoula Chatzilazarou Digitally signed by Archontoula Chatzilazarou Date: 2023.09.05 21:45:02 +03'00'
3	**Λαλάς Σταύρος**	STAVROS LALAS 18/09/2023 16:06

Μέλη Επταμελούς Εξεταστικής Επιτροπής

Α/Α	ΟΝΟΜΑ ΕΠΩΝΥΜΟ	ΥΠΟΓΡΑΦΗ
4	**Ιωάννης Παρασκευόπουλος**	Ioannis Paraskevopoulos Digitally signed by Ioannis Paraskevopoulos Date: 2023.09.11 17:39:40 +03'00'
5	**Αικατερίνη Σκουρολιάκου**	Aikaterini Skouroliakou Digitally signed by Aikaterini Skouroliakou Date: 2023.09.17 12:55:25 +03'00'
6	**Δημήτρης Μακρής**	DIMITRIOS MAKRIS 14/09/2023 09:14
7	**Παναγιώτης Αραπίτσας**	Panagiotis Arapitsas Firmato digitalmente da Panagiotis Arapitsas Data: 2023.09.08 13:13:00 +02'00'

Η έγκριση της διδακτορικής διατριβής από το Τμήμα Οίνου Αμπέλου και Ποτών του Πανεπιστημίου Δυτικής Αττικής δεν υποδηλοί αποδοχή των γνωμών του συγγραφέα (Ν. 5343/32, Άρθρο 202).

ΔΗΛΩΣΗ ΣΥΓΓΡΑΦΕΑ ΔΙΔΑΚΤΟΡΙΚΗΣ ΔΙΑΤΡΙΒΗΣ

Ο κάτωθι υπογεγραμμένος Ντουρτόγλου Γεώργιος, του Βασίλειου, υποψήφιος διδάκτορας του Τμήματος Επιστημών Οίνου, Αμπέλου & Ποτών της Σχολής επιστήμων τροφίμων του Πανεπιστημίου Δυτικής Αττικής, δηλώνω ότι:

«Είμαι συγγραφέας και δικαιούχος των πνευματικών δικαιωμάτων επί της διατριβής και δεν προσβάλλω τα πνευματικά δικαιώματα τρίτων. Για τη συγγραφή της διδακτορικής μου διατριβής δεν χρησιμοποίησα ολόκληρο ή μέρος έργου άλλου δημιουργού ή τις ιδέες και αντιλήψεις άλλου δημιουργού χωρίς να γίνεται αναφορά στην πηγή προέλευσης (βιβλίο, άρθρο από εφημερίδα ή περιοδικό, ιστοσελίδα κ.λπ.). Επίσης, βεβαιώνω ότι αυτή η εργασία έχει συγγραφεί από μένα αποκλειστικά και αποτελεί προϊόν πνευματικής ιδιοκτησίας τόσο δικής μου, όσο και του Ιδρύματος.

Παράβαση της ανωτέρω ακαδημαϊκής μου ευθύνης αποτελεί ουσιώδη λόγο για την ανάκληση του διδακτορικού διπλώματος μου».

Ντουρτόγλου Γεώργιος

Δημοσιεύσεις που περιλαμβάνονται στην διατριβή

Η παρούσα διδακτορική διατριβή βασίζεται στις παρακάτω δημοσιεύσεις

I. Ntourtoglou, G., Tsapou, E. A., Drosou, F., Bozinou, E., Lalas, S., Tataridis, P., & Dourtoglou, V. (2020). Pulsed Electric Field Extraction of α and β-Acids From Pellets of *Humulus lupulus* (Hop). *Frontiers in Bioengineering and Biotechnology*, *8:297*. https://doi.org/10.3389/fbioe.2020.00297

II. Tsapou, E. A., Ntourtoglou, G., Drosou, F., Tataridis, P., Dourtoglou, T., Lalas, S., & Dourtoglou, V. (2020). In situ Creation of the Natural Phenolic Aromas of Beer: A Pulsed Electric Field Applied to Wort-Enriched Flax Seeds. *Frontiers in Bioengineering and Biotechnology*, *8:583617*. https://doi.org/10.3389/fbioe.2020.583617

III. Pappas, V. M., Lakka, A., Palaiogiannis, D., Bozinou, E., Ntourtoglou, G., Batra, G., Athanasiadis, V., Makris, D. P., Dourtoglou, V. G., & Lalas, S. I. (2021). Use of pulsed electric field as a low-temperature and high-performance "green" extraction technique for the recovery of high added value compounds from olive leaves. *Beverages*, *7(3)*, *45*. https://doi.org/10.3390/beverages7030045

IV. Pappas, V. M., Lakka, A., Palaiogiannis, D., Athanasiadis, V., Bozinou, E., Ntourtoglou, G., Makris, D. P., Dourtoglou, V. G., & Lalas, S. I. (2021). Optimization of pulsed electric field as standalone "green" extraction procedure for the recovery of high value-added compounds from fresh olive leaves. *Antioxidants*, *10(10)*, *1554*. https://doi.org/10.3390/antiox10101554

V. Ntourtoglou, G. v., Drosou, F., Enoch, Y., Tsapou, E. A., Bozinou, E., Athanasiadis, V., Chatzilazarou, A., Dourtoglou, E. G., Lalas, S. I., & Dourtoglou, V. G. (2021). Extraction of volatile aroma compounds from toasted oak wood using pulsed electric field. *Journal of Food Processing and Preservation*, *45*(6)*:e15577*. https://doi.org/10.1111/jfpp.15577

VI. Athanasiadis, V., Lakka, A., Palaiogiannis, D., Pappas, V. M., Bozinou, E., Ntourtoglou, G., Makris, D. P., Dourtoglou, V. G., & Lalas, S. I. (2021). Pulsed electric field and

Salvia officinalis l. Leaves: A successful combination for the extraction of high value added compounds. *Foods*, *10*(9), *2014*. https://doi.org/10.3390/foods10092014

VII. Ntourtoglou, G., Drosou, F., Dourtoglou, V. G., Athanasiadis, V., Chatzimitakos, T., Bozinou, E., & Lalas, S. I. (2022). Hyphenated Extraction of Valuable Compounds from *Aesculus carnea*: Ultrasound Extraction with Pulsed Electric Field Pretreatment. *AgriEngineering*, *4*(4), 847–854. https://doi.org/10.3390/agriengineering4040054

VIII. Ntourtoglou, G., Drosou, F., Chatzimitakos, T., Athanasiadis, V., Bozinou, E., Dourtoglou, V. G., Elhakem, A., Sami, R., Ashour, A. A., Shafie, A., & Lalas, S. I. (2022). Combination of Pulsed Electric Field and Ultrasound in the Extraction of Polyphenols and Volatile Compounds from Grape Stems. *Applied Sciences*, *12*(12), 6219. https://doi.org/10.3390/app12126219

IX. Tsapou, E. A., Ntourtoglou, G., Drosou, F., Tataridis, P., Lalas, S., & Dourtoglou, V. (2022). Pulsed electric field: A “green” extraction technology for biomolecular products from glycerol with fermentation of non-Saccharomyces yeasts. *Front. Bioeng. Biotechnol. 10:964174.* https://doi.org/10.3389/fbioe.2022.964174

Τα πειράματα που περιγράφονται στην παρούσα διδακτορική διατριβή πραγματοποιήθηκαν στα:

Τμήμα Επιστημών Οίνου, Αμπέλου και Ποτών - Πανεπιστήμιο Δυτικής Αττικής

Τμήμα Επιστήμης Τροφίμων και Διατροφής - Πανεπιστήμιο Θεσσαλίας

Περίληψη

Στόχος της διατριβής αυτής είναι να διερευνήσει αν η ηλεκτροδιαπερατότητα που προκαλεί η τεχνική της PEF στα μικροβιακά, φυτικά ή και ζωικά κύτταρα είναι δυνατόν να χρησιμοποιηθεί για την εκχύλιση. Μέχρι στιγμής η τεχνική της PEF χρησιμοποιούνταν κυρίαρχα για την αποστείρωση ή γενικά τη διάσπαση του κυττάρου. Με τη χρήση της PEF επιδιώκεται η απομόνωση σημαντικών ενώσεων από διάφορους ιστούς, οι οποίες υπό άλλες συνθήκες θα απαιτούσαν μεγαλύτερους χρόνους και μεγαλύτερη ενέργεια για να απομονωθούν ή θα καταστρέφονταν.

Είναι προφανές ότι δεν θα μπορούσε να μελετηθεί το σύνολο των ενώσεων που επηρεάζονται από την PEF. Έτσι, σημαντικές ενώσεις θα θεωρήσουμε τις αρωματικές ενώσεις που εκχυλίζονται στα ποτά κατά τη ζύμωση από το περιβάλλον τους, όπως π.χ. από το ξύλο βαρελιού ή από τους μικροοργανισμούς που συμμετέχουν στη ζύμωση ή από πρόσθεση φυτικών ιστών που παραδοσιακά χρησιμοποιούνται στα ποτά, όπως ο λυκίσκος στον ζύθο. Θα θεωρήσουμε ακόμα τις φαινολικές ουσίες που έχουν ιδιαίτερο ενδιαφέρον για την ανθρώπινη υγεία όπως κατεχίνες, τανίνες, ανθοκυανίνες οι οποίες συναντώνται στα υποπροϊόντα της οινοποιίας, όπως τα στέμφυλα και οι βόστρυχοι. Τέλος, θα μελετηθούν ευεργετικές για τον άνθρωπο ουσίες που πρόσφατα μπήκαν στην ανθρώπινη διατροφή, όπως τα φαινολικά των φύλλων της ελιάς.

Σκοπός ήταν να αποδειχθεί ότι η νέα αυτή τεχνική μπορεί να συγκαταλεχθεί ανάμεσα στις κλασικές τεχνικές εκχύλισης και απομόνωσης ουσιών είτε από φυτικούς ιστούς είτε από ζυμωμένα τρόφιμα και ποτά είτε από βιοτεχνολογικές διεργασίες. Επίσης, ότι μπορεί να αυξήσει τις αποδόσεις των εκχυλίσεων με έναν τρόπο ήπιο και μη καταστροφικό. Εν κατακλείδι, θα μπορούσαμε να συνοψίσουμε ότι η τεχνική αυτή αποτελεί μια νέα τεχνική απομόνωσης που μπορεί να προστεθεί στις βιομηχανικές πρακτικές.

Επίσης, ένας **δευτερεύων σκοπός** ήταν να δειχθεί ότι η τεχνική αυτή μπορεί να εφαρμοστεί με έναν νέο εξοπλισμό τεχνολογικά, πιο απλό, χωρίς να απαιτούνται ιδιαίτερα ηλεκτρικά πεδία και συνεπώς, λιγότερο απαιτητικό, ενεργειακά**.**

Η τεχνική του παλλόμενου ηλεκτρικού πεδίου όπως θα αναλυθεί στην παρούσα διατριβή χωρίζεται σε τμήματα, ηλεκτρικά, μηχανικά και αναλυτικά, πάντα με στόχο την εκχύλιση μεταβολιτών. Επίσης μέσα από αυτό τον διαχωρισμό επιτεύχθηκε και η ελαχιστοποίηση των αναγκών στα επιμέρους τμήματα της δημιουργίας του πεδίου, έτσι ώστε να μπουν οι βάσεις για να μπορεί να κατασκευαστεί και να χρησιμεύσει στη βιομηχανία.

Η ηλεκτρική πλευρά χωρίζεται με τη σειρά της σε 3 διαφορετικές ενότητες:

1) Σχεδιασμός κυκλωμάτων που μπορούν να παράγουν ηλεκτρικούς παλμούς από 1000V έως 5000V για παλμική εκχύλιση με τη χρήση ηλεκτρικού πεδίου.

2) Κατασκευή μικροτεχνικού ηλεκτροδίου που διευκολύνει την εκχύλιση.

3) Διεξαγωγή εκχυλίσεων για να αποδειχθεί ότι το κύκλωμα και το ηλεκτρόδιο είναι σε λειτουργία.

Το σχεδιασμένο ηλεκτρόδιο κατέχει καθοριστικό ρολό στην αποτελεσματικότητα της εκχύλισης κατά την εκτέλεση της διαδικασίας. Επομένως, η επιλογή υλικού και ο σχεδιασμός είναι κομβικής σημασίας για τη μετέπειτα πορεία.

Από πλευράς εκχύλισης φυτικών ιστών, η διατριβή αυτή ξεκίνησε από τον λυκίσκο και τη χρήση του στην μπύρα. Στον λυκίσκο μελετήθηκε η εκχυλισιμότητα από το φυτικό υλικό του, των πικρικών α- και β- οξέων, όπως η χουμουλόνη και η λουπουλόνη, καθώς και τα πτητικά αρωματικά, όπως το καρυοφυλλένιο και τα λοιπά τερπενικά. Η μελέτη έδειξε ότι το ΠΗΠ μπορεί να βελτιώσει την εκχυλισιμότητα των α-οξέων έως και 20%, ενώ συνέβαλε στην αύξηση του καρυοφυλλένιου και των τερπενίων της τάξης του 6%. Μελετώντας πάντα την μπύρα, η μελέτη επεκτάθηκε και σε ενώσεις που προέρχονται από βιοχημικά μονοπάτια με βιομετατροπές, όπως τα φαινολικά αρώματα, η 4-βινυλ γουαικόλη (4-VG), ένα παράγωγο του φερουλικού οξέος και παράγωγα του ξύλου, όπως η βανιλίνη, η συρινγκαλδεύδη, η λακτόνη δρυός και η φουρφουράλη σε πρότυπα διαλύματα. Η χρήση του ΠΗΠ οδήγησε σε αύξηση των πτητικών κατά 234% μετά την εκχύλιση σε σχέση με τα δείγματα που δεν είχαν υποστεί επεξεργασία με ΠΗΠ .

Η τεχνική της PEF μελετήθηκε για εκχυλίσεις σε διαδικασίες μετά-ζυμωτικές και επεκτάθηκε σε υποπροϊόντα με σημαντικό περιεχόμενο σε βιοενεργές ουσίες, όπως οι βόστρυχοι, τα φύλλα της ελιάς, το φασκόμηλο και η αγριοκαστανιά. Από τα φύλλα της ελιάς εκχυλίστηκε ο ρουτινοζίτης της κουερκετίνης και της απιγενίνης, ο γλυκοζίτης στις θέσεις 7 και 3 της λουτεολίνης και η ολεοπαίνη. Η βέλτιστη συμβολή του ΠΗΠ στην ικανότητα εκχύλισης ολικών πολυφαινολών βρέθηκε στο 38% με 117% για συγκεκριμένους μεταβολίτες. Τέλος, στην αγριοκαστανιά μελετήθηκε η επίδραση στην εκχύλιση του νεο χλωρογενικού οξέος (Neochlorogenic acid) της καμφερόλης και των γλυκοζιτών της, της κουερκετίνης και του γαλακτοζίτη της. Υπό βέλτιστες συνθήκες, σύμφωνα με τα αποτελέσματα, μπορούν να εξαχθούν έως και 33% περισσότερες φαινολικές ενώσεις σε σχέση με τα μη επεξεργασμένα δείγματα.

Λέξεις κλειδιά: Παλλόμενο ηλεκτρικό πεδίο, στελέχη ζυμών, εκχύλιση, βελτιστοποίηση, βιωσιμότητα

Abstract

The aim of this thesis is to investigate whether the electropermeability caused by the PEF technique in microbial, plant or even animal cells can be used for extraction. So far, the PEF technique has been predominantly used for sterilization, or in general, cell disruption. The use of PEF seeks to isolate important compounds from various tissues, which under other conditions would require longer times and more energy to be isolated or be destroyed.

It is obvious that not all compounds affected by PEF could be studied. So we will consider important the aromatic compounds that are extracted in the drinks during fermentation from their environment such as the wood of the barrel, or from the microorganisms involved in fermentation, or from the addition of plant tissues traditionally used in beverages such as hops in beer. We will also consider the phenolic substances that are of particular interest to human health such as catechins, tannins, anthocyanins which are found in the by-products of winemaking, such as grapevines and marcs. Finally, beneficial substances for humans that have recently entered the human diet, such as olive leaf phenolics, will be studied.

The aim was to demonstrate that this new technique can be included among the classic techniques for the extraction and isolation of substances, either from plant tissues or from fermented foods and drinks, or for biotechnological processes. Also, that it can increase the yields of extractions in a gentle and non-destructive way. In conclusion we could summarize that this technique is a new isolation technique that can be added to industrial practices.

Also, a secondary purpose was to show that this technique can be applied with a equipment, which is simpler without requiring special electric fields and therefore, less demanding, energetically.

The pulsed electric field technique as will be analyzed in this thesis is divided into sections, electrical, mechanical and analytical always with the aim of extracting metabolites. Also, through this separation, the minimization of the needs in the individual parts of the creation of the field was achieved, so that the foundations could be laid for it to be built and used in industry.

The electrical side is in turn divided into 3 different sections.

1) Design of circuits that can generate electric pulses from 1000V to 5000V for pulse extraction using an electric field.

2) Construction of a microtechnical electrode that facilitates the extraction.

3) Conduct extractions to prove that the circuit and electrode are operational.

The designed electrode plays a decisive role in the extraction efficiency during the execution of the process. Therefore, the choice of material and the design are of crucial importance for the subsequent course.

In terms of extracting plant tissues, this thesis started with hops and their use in beer. In hops, the extractability from its plant material of bitter α- and β-acids such as humulone and lupulone as well as volatile aromatics such as caryophyllene and other terpenes was studied. The study showed that PEF can improve the extractability of α-acids up to 20% while it contributed to the increase of caryophyllene and terpenes by 6%. Studying the use of PEF in beer making, the study also extended to compounds derived from biochemical pathways with biotransformations such as phenolic aromas 4-vinyl guaicol (4-VG) a derivative of ferulic acid, and wood derivatives such as vanillin, syringaldedus, oak lactone, and furfural in standard solutions. The use of PDP resulted in a 234% increase in volatiles after extraction compared to samples not treated with PDP.

The PEF technique was studied for extractions in post-fermentation processes and was extended to by-products with a significant content of bioactive substances such as boletus, olive leaves, sage and horse chestnut. The rutinoside of quercetin and apigenin, the glucoside at positions 7 and 3 of luteolin and oleopain were extracted from olive leaves. The optimal contribution of PIP to the extraction capacity of total polyphenols was found to be 38% with 117% for specific metabolites. Finally, in the horse chestnut, the effect on the extraction of the neochlorogenic acid (Neochlorogenic acid) of kaempferol and its glycosides, quercetin and its galactoside was studied. Under optimal conditions, according to the results, up to 33% more phenolic compounds can be extracted compared to untreated samples.

Keywords: Pulsed electric field, yeast strain, extraction, optimization, sustainability

Συντομεύσεις, ακρωνύμια, σύμβολα και ορισμοί

PEF	Pulsed electric field
ΠΗΠ	Παλλόμενο ηλεκτρικό πεδίο
ΤΜ	Τάση μεμβράνης
IGBT	Insulated Gate Bipolar Transistor
MOSFET	Metal oxide semiconductor field-effect transistor
PE	Πολυαιθυλένιο
PP	Πολυπροπυλένιο
ODP	Παλμοί ταλάντωσης με αποσύνθεση
EDP	Εκθετικοί παλμοί με αποσύνθεση
S.c	*Saccharomyces cerevisiae*
ΥΥΠ	Υψηλή Υδροστατική Πίεση
HPCD	High pressure carbon dioxide
ΥΠΔΑ	Υψηλής πίεσης διοξείδιο του άνθρακα
PAA	Peracetic acid
SW	Square wave
PNP	Positive-Negative-Positive
4-VG	4-Βινυλγουαϊακόλη

Περιεχόμενα

Περιεχόμενα εικόνων

Περιεχόμενα πινάκων

Αντί Προλόγου

RESOLUTION OIV-OENO 634-2020

TREATMENT OF GRAPES BY PULSED ELECTRIC FIELDS - (PEF) THE GENERAL ASSEMBLY,

OIV-OENO 634-2020

TITLE:

TREATMENT OF GRAPES BY PULSED ELECTRIC FIELDS (PEF)

DEFINITION:

A process that consists of the application of sufficiently high pulsed electric fields (PEF) to destemmed and crushed grapes that causes the permeabilization of the cell membranes especially of the grape skins.

OBJECTIVES:

Treatment of red grapes destemmed and crushed by PEF to

Facilitate and increase the extraction of valuable substances such as polyphenols, yeast available nitrogen, aroma compounds including precursors, and other substances located inside the grape cells.

Reduce maceration time.

Treatment of white grapes destemmed and crushed by PEF to facilitate and increase the extraction of valuable substances such as yeast available nitrogen, aroma compounds including precursors, and other substances located inside the grape cells.

PRESCRIPTIONS:

The technique consists of the application of pulsed electric fields in the range of nanoseconds to milliseconds which are sufficiently high to permeabilize the cell membranes. The destemmed and crushed grapes are treated in at least one treatment chamber with at least one pair of electrodes.

OIV recommendation:

Admitted.

Certified in conformity Paris-videoconference, 26th November 2020

The General Director of the OIV Secretary of the General Ass

Ο OIV στη συνεδρία της εικοστής 26 Νοεμβρίου 2020 αποφάσισε την αποδοχή της τεχνικής του παλλόμενου ηλεκτρικού πεδίου για την επεξεργασία των σταφυλιών προκειμένου να επιτευχθούν τα παρακάτω αποτελέσματα.

α) Επεξεργασία κόκκινων σταφυλιών με ΠΗΠ που έχουν προηγουμένως αποβοστρυχωθεί και θρυμματιστεί, προκειμένου να επιτευχθεί η αύξηση οινολογικών πολύτιμων ουσιών, όπως πολυφαινόλες, άζωτο διαθέσιμο για τη μαγιά, αρωματικές ενώσεις συμπεριλαμβανομένων πρόδρομων ουσιών και άλλες ουσίες που βρίσκονται μέσα στα κύτταρα του σταφυλιού.

β) Επεξεργασία λευκών σταφυλιών που έχουν αποσταλεί και θρυμματιστεί από ΠΗΠ προκειμένου να διευκολυνθεί η εκχύλιση πολύτιμων ουσιών, όπως το διαθέσιμο άζωτο της ζύμης, οι αρωματικές ενώσεις συμπεριλαμβανομένων των πρόδρομων ουσιών και άλλες ουσίες που βρίσκονται μέσα στα κύτταρα του σταφυλιού.

Το εργαστήριο της Βιοργανικής Χημείας του Τμήματος Επιστημών Οίνου, Αμπέλου & Ποτών του Πανεπιστήμιου Δυτικής Αττικής ασχολείται από το 2006 με αυτήν την τεχνική, κατασκευάζοντας και εφαρμόζοντας διάφορα συστήματα που έχουν σαν στόχο την εκχύλιση πολύτιμων συστατικών που αφορούν τον χώρο της οινολογίας, τον χώρο των προσθέτων υπό προϊόντων (στέμφυλων) και λοιπών άλλων και τον χώρο της βυνοποίησης.

Το 2016 στο παγκόσμιο συνέδριο του OIV παρουσιάζει για πρώτη φορά την τεχνική αυτή, εφαρμοσμένη στην ταχεία παλαίωση κρασιών.(Drosou et al., 2017a)

Ο στόχος ήταν να ευαισθητοποιηθεί η παγκόσμια ερευνητική και παραγωγική κοινότητα η οποία δραστηριοποιείται γύρω από αυτόν τον χώρο, έτσι ώστε, να αποδεχθεί και ναδεχθεί την τεχνική αυτή η οποία είναι πάρα πολύ απλή σαν μια επίσημη τεχνική εκχύλισης, απομόνωσης και αποστείρωσης.

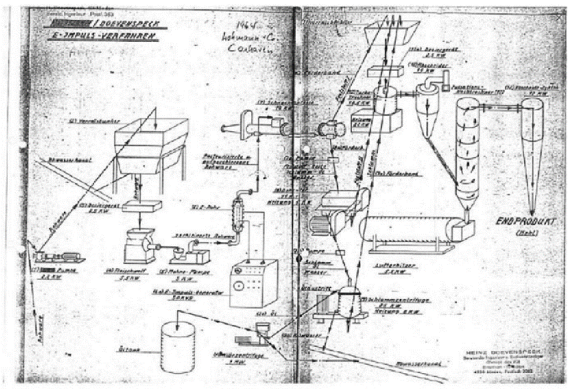
Η απόφαση αυτή του OIV άνοιξε το δρόμο για την εφαρμογή μιας τεχνικής η οποία είχε προηγουμένως μελετηθεί για άλλους στόχους, καθώς και για τη χρήση αυτής της τεχνικής προκειμένου να επιτευχθεί ποιοτικώς, καλύτερη παραλαβή μούστου σε συντομότερο χρονικό διάστημα και χωρίς επίπονες τεχνικές.

Φυσικά, η απόφαση αυτή του ΟΙV δεν άφησε μόνο το πεδίο ελεύθερο στην οινολογία, αλλά οδήγησε και στην εφαρμογή των ΠΗΠ σε πολλά άλλα φυτικά μέρη, αλλά και σε πολλά άλλα ζυμωμένα τρόφιμα ή ποτά και επεκτάθηκε στην αποστείρωση, στην εκχύλιση σημαντικών ουσιών από φύλλα και καρπούς, από υποπροϊόντα της οινοποίησης, όπως στέμφυλα και βόστρυχοι, καθώς και άλλα.

1. Ιστορική αναδρομή

Εικόνα 1::Heinz Doevenspeck

Από την προϊστορική εποχή έχουν καταγραφεί προσπάθειες του ανθρώπινου γένους να διατηρεί τις τροφές του, αλλά και να τις βελτιώνει. Στο πέρασμα των αιώνων διάφορες τεχνικές αναπτυχθήκαν και προς τις δυο κατευθύνσεις. Με την έλευση του ηλεκτρισμού τόσο οι μέχρι τότε παραδοσιακές μορφές εξελίχθηκαν όσο και νέες αρχίσαν να αναπτύσσονται (Boarder & Masood, 2019). Σε αυτό το πλαίσιο στα μέσα του 20ου αιώνα άρχισε να αναπτύσσεται η τεχνική του παλλομένου ηλεκτρικού πεδίου από τον Γερμανό Heinz Doevenspeck (1917–1993) που αν και αυθαίρετα από πολλούς θεωρείται ο πατέρας των ΠΗΠ. Λίγα είναι γνωστά για την πρώιμη ζωή του πέρα από το ότι τραυματίστηκε στον δεύτερο παγκόσμιο πόλεμο και ότι αποφοίτησε από άγνωστο πανεπιστήμιο σαν Μηχανολόγος Μηχανικός. Παρόλα αυτά, το 1960 καταθέτει στο γερμανικό επιμελητήριο πατεντών την πατέντα με αριθμό DE1237541B που θεωρείται το παλαιότερο καταγεγραμμένο παράδειγμα ΠΗΠ (HEINZ, 1960.). Αξιολογώντας την αποτελεσματικότητα της πατέντας του, συνεργάζεται με μια τοπική εταιρία αλιευμάτων για την κατασκευή μιας πρότυπης μονάδας επεξεργασίας ψαριών. Πειράματα που πραγματοποιήθηκαν σε ωοτόκα πουλερικά έδειξαν ότι οι τροφές που ήταν επεξεργασμένες με τα ΠΗΠ και παράγονταν από φτωχότερα σε πρωτεΐνες ιχθυάλευρα ήταν πολύ καλύτερες από τα συμβατικά ιχθυάλευρα από το Περού, όσον αφορά την απόδοση του μεταβολισμού της ζωοτροφής για το συγκεκριμένο είδος. Στην εικόνα 2 φαίνεται η γραμμή παραγωγής της ιχθυοκαλλιέργειας. Η πηγή θερμικής επεξεργασίας έχει αντικατασταθεί με ένα σύστημα ΠΗΠ. Επίσης, κατά τη διάρκεια πειραμάτων που πραγματοποίησε εκθέτοντας το βακτήριο Escherichia coli σε

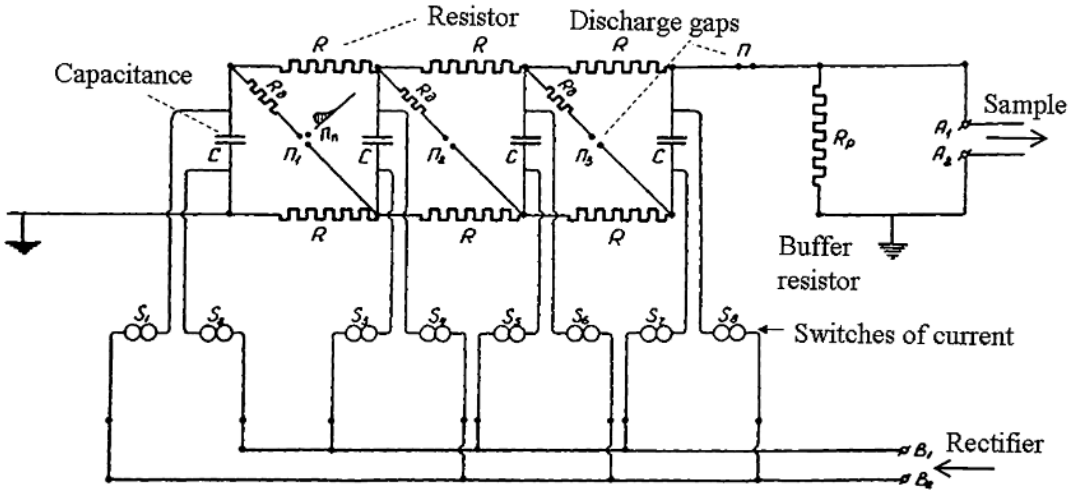


(Πρωτότυπο διάγραμμα από Doevenspeck 1964)

ΠΗΠ, παρατήρησε ότι όταν το πεδίο είναι κάτω από 3KV/cm τότε ο ρυθμός ανάπτυξης επιταχύνεται, ενώ όταν τα ξεπερνάει, ο ρυθμός ανάπτυξης τους μειώνεται είτε τα βακτήρια πεθαίνουν (Sitzmann et al., 2017).

Εικόνα 3:Zagorul'ko

Ταυτόχρονα σχεδόν με τον Doevenspeck, δύο νεαροί Ουκρανοί επιστήμονες μηχανικής τροφίμων, οι Zagorul'ko και Flaumenbaum ξεκινούν τις δραστηριότητές τους για την εφαρμογή της ηλεκτρικής ενέργειας στη βιομηχανία τροφίμων το 1949. Ο Zagorul'ko μεταξύ του 1949 και του 1953 πραγματοποιεί πρωτοποριακά πειράματα με συνεχές και εναλλασσόμενο ρεύμα και παρατηρεί την καταστροφή των κυτταρικών μεμβρανών, μια διαδικασία που την ονομάζει ηλεκτροπλασμόλυση. Καθώς τα πειράματα του συνεχίζονται, καταφέρνει να παραλάβει χυμό ζαχαρότευτλων σε θερμοκρασία δωμάτιου, μια διαδικασία που θεωρείται επαναστατική για εκείνη την εποχή. Συνεχίζοντας την ερευνά του μεταξύ του 1953 και του 1957, προτείνει την χρήση των ΠΗΠ για να πέτυχει ηλεκτροπλασμόλυση. Προτείνει ένα σύστημα που έχει διάρκεια παλμού 20 μs και ένταση πεδίου 20 kV/s, ένα σύστημα που θυμίζει έντονα τα σύγχρονα (Sitzmann et al., 2017; Vorobiev & Lebovka, 2020).



Εικόνα 4:Σχέδιο γεννήτριας παλμών που κατασκευάστηκε από τον Zagorul'ko το 1955-1957 (Zagorul'ko, 1958)

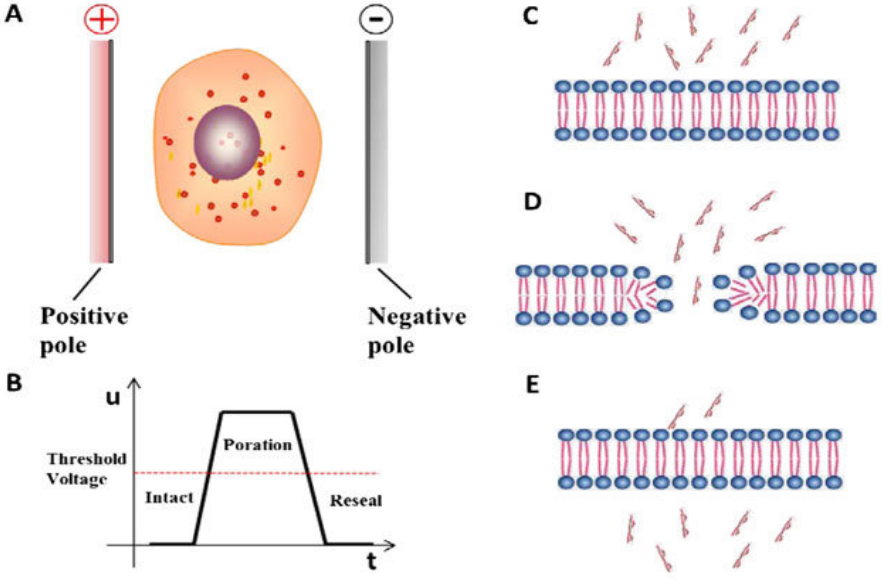
Ο δεύτερος Ουκρανός μηχανικός τροφίμων, ο Flaumenbaum, την ίδια περίοδο ξεκινάει την έρευνά του πάνω στη μείωση του χρόνου εκχύλισης διάφορων φρούτων και

λαχανικών. Το 1949 δημοσιεύει την πρώτη του εργασία πάνω στην επίδραση του ηλεκτρικού φορτιού σε σταφύλια μήλα και καρότα. Στην συνέχεια, συνεργάζεται με τον μηχανικό Yablochnik για να δηλώσουν την πατέντα, αλλά και να κατασκευάσουν ένα βιομηχανικής κλίμακας σύστημα ηλεκτροπλασμόλυσης. Η συγκεκριμένη εγκατάσταση δοκιμάστηκε σε εργοστάσια φρούτων και σε διαφορά οινοποιεία.(Vorobiev & Lebovka, 2020)

2. Ηλεκτροδιάτρηση

Η ηλεκτροδιάτρηση (electroporation) είναι ένα βιοφυσικό φαινόμενο κατά το οποίο η διαπερατότητα της κυτταρικής μεμβράνης αυξάνεται. Υπάρχουν δυο είδη ηλεκτροδιάτρησης. Η η αναστρέψιμη που λαμβάνει χώρα όταν η διαπερατότητα της κυτταρικής μεμβράνης είναι προσωρινή και τα επεξεργασμένα κύτταρα επιβιώνουν. Η ηλεκτροδιάτρηση που οδηγεί σε κυτταρικό θάνατο είναι γνωστή και ως μη αναστρέψιμη ηλεκτροδιάτρηση (Golberg & Rubinsky, 2013).

Η πρώτη αναφορά σε αυτό που σήμερα ονομάζεται ηλεκτροδιάτρηση γίνεται το 1754 από τον J.A. Nollet. Ο πρώτος παρατήρησε ότι όταν ανθρώπινος ή ζωικός ιστός εκτίθεται σε ηλεκτρικό πεδίο, τότε εμφανίζονται κόκκινες κηλίδες. Με την πρόοδο της επιστήμης κατά τον 20ο αιώνα, οι κλάδοι που ασχολούνταν με την ηλεκτροδιάτρηση γνωρίσαν ραγδαία επιστημονική πρόοδο (Rolong et al., 2017). Μια από της σημαντικότερες δουλειές που καταγράφονται είναι του Dr Zimmermann στην οποία περιγράφεται η χρήση της αναστρέψιμης ηλεκτροδιάτρησης για την σύντηξη μεταξύ των κυττάρων (Zimmermann, 1982).



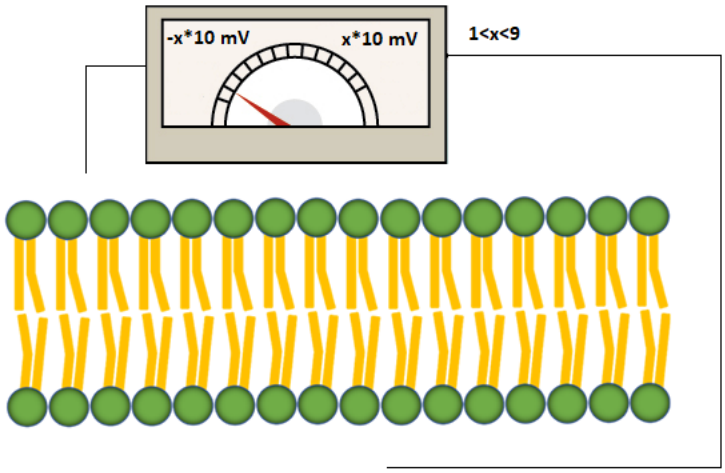
Εικόνα 5:Βασική αρχή ηλεκτροδιάτρησης(Du et al., 2018)

Το φαινόμενο της ηλεκτροδιάτρησης έχει απασχολήσει διάφορους επιστήμονες από πολλούς κλάδους μιας και βρίσκει εφαρμογές στην ιατρική, στη βιολογία, στην τεχνολογία τροφίμων και αλλού (Carter & Shieh, 2015;

Tylewicz, 2020; Wei et al., 2021).

2.1 Ηλεκτροδιαπερατότητα σε επίπεδο κυττάρου

Σχεδόν όλα τα κύτταρα διατηρούν μια διαφορά ηλεκτρικού δυναμικού μεταξύ της εσωτερικής και της εξωτερικής πλευράς της μεμβράνης πλάσματος. Αυτή η διαφορά δυναμικού δημιουργείται και ρυθμίζεται από ένα σύστημα καναλιών ιόντων και ονομάζεται τάση μεμβράνης (TM). Στα ευκαρυωτικά κύτταρα, η τάση ηρεμίας τυπικά κυμαίνεται από – 40 έως – 70 mV. Το αρνητικό πρόσημο δείχνει την κατεύθυνση του πεδίου, δηλαδή ότι το εσωτερικό δυναμικό είναι χαμηλότερο από το εξωτερικό. Καθώς αυτή είναι η φυσική κατάσταση των μεμβρανών, τόσο τα λιπίδια όσο και οι πρωτεΐνες, εξελιχθήκαν, ώστε να είναι καλά προσαρμοσμένα και να λειτουργούν υπό τάσεις σε αυτό το εύρος (Gimsa & Wachner, 2001; Kotnik & Miklavčič, 2000).



Εικόνα 6: Διαφορά δυναμικού μεμβράνης

2.2 Συσχέτιση μεταξύ διαμεμβρανικής τάσης και Μεταφορές με Διαμεσολάβηση Ηλεκτροδιαπερατότητας

Η έκθεση ενός κυττάρου σε ένα εξωτερικό ηλεκτρικό πεδίο έχει ως αποτέλεσμα η διαφορά τάσης στην κυτταρική μεμβράνη κατά τη διάρκεια έκθεσης να είναι ανάλογη με την ένταση του εξωτερικού ηλεκτρικού πεδίου (Kotnik et al., 1998). Έτσι, οι εκθέσεις σε αρκετά ισχυρά πεδία μπορούν να προκαλέσουν TM που υπερβαίνει κατά πολύ το

εύρος ηρεμίας της και προκαλεί τόσο δομικές αλλαγές στη μεμβράνη όσο και αλλαγές στα δομικά της μόρια που δεν μπορούν να συμβούν υπό φυσιολογικές συνθήκες.

Μεταξύ των πιο ξεκάθαρων και πολλά υποσχόμενων τέτοιων επιδράσεων είναι η ηλεκτροδιαπερατότητα (electropermeabilization) της μεμβράνης. Μια ταχεία και ουσιαστική αύξηση της διαπερατότητας της μεμβράνης, που γίνεται αντιληπτή από τη διαμεμβρανική μεταφορά μορίων για τα οποία μια άθικτη μεμβράνη είναι πρακτικά αδιαπέραστη (Pucihar et al., 2008; Yarmushet al., 2014).

2.3 Κινητική της Ηλεκτροδιαπερατότητας

Η κύρια συνέπεια της ηλεκτροδιαπερατότητας της μεμβράνης είναι η εισροή μορίων στο κύτταρο, που υπό φυσιολογικές συνθήκες δεν θα μπορούσαν να διαπεράσουν την μεμβράνη, και η εκροή βιομορίων από το κύτταρο. Η κινητική της διαμεμβρανικής μεταφοράς που λαμβάνει χώρα λόγω της ηλεκτροδιαπερατότητας, έχει μελετηθεί εκτενώς, αποκαλύπτοντας ότι η ηλεκτρική αγωγιμότητα και η διαπερατότητα της μεμβράνης αυξάνονται ανιχνεύσιμα σε λιγότερο από ένα μικροδευτερόλεπτο (μs) μετά την έναρξη του ηλεκτρικού παλμού, με την προϋπόθεση ότι η ΤΜ υπερβαίνει μια ορισμένη «κρίσιμη» τιμή. Ο ορός κρίσιμη τιμή εδώ χρησιμοποιείται καταχρηστικά μιας και δεν είναι καθολική σταθερά, αλλά μεταβλητή που εξαρτάται από μια σειρά παραγόντων. Η κινητική της διαμεμβρανικής μεταφοράς μπορεί χονδρικά να χωριστεί σε πέντε στάδια, όπως συνοψίζονται στον Πίνακα 1: η έναρξη της διαπερατής κατάστασης, η επέκτασή της, η σταθεροποίηση με μερική ανάκτηση, η επανασφράγιση της μεμβράνης και τελικά, η σταδιακή διακοπή αυτών που αναφέρονται ως υπολειπόμενα αποτελέσματα μνήμης που αντικατοπτρίζονται στις αλλοιωμένες φυσιολογικές διεργασίες και αντιδράσεις των κυττάρων σε διάφορους στρεσογόνους παράγοντες.

Πίνακας 1 Στάδια διαμεμβρανικής τάσης σε σχέση με τον χρόνο (Kotnik et al., 2019)

Στάδιο	Χρονικό προφίλ
Έναρξη: Έναρξη της ηλεκτρικής αγωγιμότητας και διαπερατότητας της μεμβράνης που αυξάνεται ανιχνεύσιμα όταν η τάση μεμβράνης (ΤΜ) υπερβαίνει την «κρίσιμη» τιμή.	Νανοδευτερόλεπτα (αγωγιμότητα) Μικροδευτερόλεπτα (διαπερατότητα)
Επέκταση: Όσο η ΤΜ παραμένει πάνω από την «κρίσιμη» τιμή, η αγωγιμότητα και η διαπερατότητα παραμένουν και/ή εντείνονται.	Μέχρι το τέλος του παλμού
Μερική ανάκτηση: Αφού το ΤΜ πέσει κάτω από την «κρίσιμη» τιμή, η αγωγιμότητα και η διαπερατότητα μειώνονται γρήγορα αλλά όχι πλήρως, σταθεροποιούνται σε ανιχνεύσιμα επίπεδα και εξακολουθούν να επιτρέπουν τη διαμεμβράνικη διάχυση ιόντων και μορίων.	Μικροδευτερόλεπτα (αγωγιμότητα) Χιλιοστά του δευτερολέπτου (διαπερατότητα)
Επανασφράγιση: Η μεμβράνη ανακτά σταδιακά το φυσιολογικό της επίπεδο διαπερατότητας (εκτός εάν η βλάβη είναι μη αναστρέψιμη και το κύτταρο χάσει τη βιωσιμότητα του).	Δευτερόλεπτα έως λεπτά
Μνήμη: Ακόμη και μετά την πλήρη επαναφορά της μεμβράνης στην αρχική της κατάσταση, το κύτταρο μπορεί να επιδεικνύει αλλοιώσεις στις φυσιολογικές διεργασίες και αντιδράσεις σε στρεσογόνους παράγοντες πριν επιστρέψει τελικά πλήρως στην κανονική του κατάσταση.	Ώρες

Από θεωρητική σκοπιά, η ηλεκτροδιαπερατότητα της μεμβράνης είτε πρόκειται για συνέπεια δομικής αναδιάταξης των λιπιδίων της είτε χημικής τροποποίησης τους ή λειτουργική ρύθμιση των πρωτεϊνών της ή συνδυασμός τους, δεν συμβαίνει αυστηρά πάνω από ένα συγκεκριμένο κατώφλι ηλεκτρικού φορτίου, αλλά εξαρτάται από διάφορους παράγοντες. Ακόμα, εμπειρικά, για κάθε τύπο κυττάρου, τύπο μορίου που μεταφέρεται, διάρκεια έκθεσης και συγκεκριμένο σύνολο συνθηκών, όπως η θερμοκρασία, υπάρχει μια κρίσιμη τιμή του πεδίου που πρέπει να ξεπεραστεί για να γίνει ανιχνεύσιμη η μεταφορά μέσω ηλεκτροδιαπερατότητας και υπάρχει μια άλλη, υψηλότερη κρίσιμη τιμή του πεδίου που δεν πρέπει να ξεπεραστεί σε περίπτωση που είναι επιθυμητή η σταθεροποίηση, ανάκτηση και επανασφράγιση της μεμβράνης.

Οι άμεσες παρατηρήσεις μέσω μικροσκοπίου αποκαλύπτουν ότι η μεταφορά μέσω ηλεκτροδιαπερατότητας εξαρτάται σε μεγάλο βαθμό από το μέγεθος και το φορτίο των μορίων. Μικρά μόρια μπορούν έτσι να εισέλθουν στο κύτταρο τόσο κατά τη διάρκεια όσο και μετά τον παλμό, και μέσω των περιοχών μεμβράνης με αρκετά χαμηλά αρνητική ΤΜ ή αρκετά υψηλή θετική ΤΜ (Kotnik et al., 2010). Για τα φορτισμένα σωματίδια, η είσοδος κατά την διάρκεια του παλμού είναι κυρίως από την πλευρά με την αντίθετη πολικότητα της ΤΜ, ενώ μετά τον παλμό η είσοδος είναι ως επί το πλείστον διάχυτη και προέρχεται και από τις δύο διάφορες δυναμικού (Pucihar et al., 2008). Μεγαλύτερα φορτισμένα μόρια εισέρχονται μόνο κατά τη διάρκεια του παλμού και μόνο από την πλευρά με την αντίθετη πολικότητα της ΤΜ (Paganin-Gioanni et al., 2011).

3. Στοιχεία της Τεχνικής Παλλόμενου πεδίου

3.1 Η κυψελίδα επεξεργασίας σαν Πυκνωτής

Παρακάτω παρουσιάζονται οι βασικές αρχές των πυκνωτών που πρέπει να ακολουθήσουμε για να αντιληφθούμε τη συνέχεια αυτής της διατριβής. Για την επεξήγηση της σχέσης κυψελίδας - πυκνωτή θα χρησιμοποιήσουμε το απλούστερο από τα σχήματα κυψελίδων που κατασκευάστηκαν. Η κυψελίδα λοιπόν αυτή αποτελούνταν από πλάκες ανοξείδωτου χάλυβα (SS 316) διαστάσεων (100 * 100) mm^2 και των οποίων η μεταξύ τους απόσταση ήταν 5 mm.

Εφαρμόζουμε στις πλάκες αυτού του πυκνωτή μια διαφορά δυναμικού ίση με 1000V. Θεωρούμε ότι ο πυκνωτής βρίσκεται υπό κενό (ε_0 η απόλυτη διηλεκτρική σταθερά του κενού: ε_0=8,85E-12 C^2/Nm^2)

τότε η χωρητικότητα τους C θα ήταν ίση σύμφωνα με τον τύπο:

$$C = -\frac{\varepsilon o * S}{4\pi d} = \frac{8.85*10^{-12}10^{-2}}{1.26*10^{-1}} = Farad = 7.05 * 10^{-13}\ \text{F}$$

Το δε φορτίο στις πλάκες του πυκνωτή θα ήταν ίσο με :

$$Q = C * V = 7.05 * 10^{-13} * 1000 = 7.05 * 10^{-10}\ \text{Coulomb}$$

Το δε πεδίο μέσα στον πυκνωτή θα ήταν ίσο με :

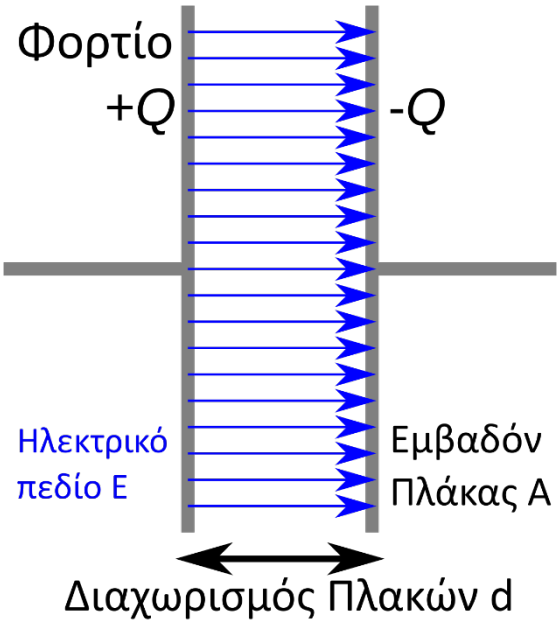
$$E = \frac{V}{d} = \frac{1000}{0{,}5 * 10^{-2}} = 2 * 10^5\ V/m$$

Απομονώνουμε τον πυκνωτή από την πηγή του ρεύματος που δίνει τη διαφορά δυναμικού των 1000V και τον απομονώνουμε έτσι, ώστε το φορτίο του να παραμείνει σταθερό. Ανάμεσα στις πλάκες του πυκνωτή τοποθετούμε ένα υλικό του οποίου η διηλεκτρική σταθερά ε είναι ίση με 4 και υπολογίζουμε πρώτα το πεδίο μέσα στο διηλεκτρικό:

$$E = \frac{\sigma}{\varepsilon_0 \varepsilon_\sigma}$$

Οπού σ η πυκνότητα του επιφανειακού φορτίου δηλαδή 7,05*10-8 C/ m^2 άρα

E=3,18*104 V/m



Εικόνα 7: Η κυψελίδα σε ρολό πυκνωτή

$$3.18 * 10^4 = \frac{V}{0.01} \rightarrow = 318\ volt$$

Από τα παραπάνω συμπεραίνεται ότι η διαφορά δυναμικού στις πλάκες του πυκνωτή, γεμισμένου με το υλικό του οποίου η διηλεκτρική σταθερά είναι 4, είναι 318 V.

Η δε χωρητικότητα αυτού του πυκνωτή του είναι ίση:

$$\frac{Q}{V} = \frac{7{,}05 * 10^{-10}}{318} = 2{,}21 * 10^{-12}\text{Farad}$$

Αυτή είναι και η συμπεριφορά της κυψελίδας μας, παρουσία ενώσεων άλλων υλικών των οποίων όμως η διηλεκτρική σταθερά διαφέρει από το ένα υλικό στο άλλο. Κατά τη διάρκεια αυτής της διατριβής, συναντήσαμε διάφορες διηλεκτρικές σταθερές, όπως αυτές των αλκοολικών δειγμάτων ή αυτές των στερεών που επρόκειτο να υποστούν εκχυλίσεις ή άλλων υποπροϊόντων των ζυμώσεων όπου περιείχαν περίπλοκα μείγματα των οποίων οι διηλεκτρικές σταθερές ήταν δύσκολο να υπολογιστούν με ακρίβεια.

Για να προχωρήσουμε στην ανάπτυξη της θεωρίας την οποία θα παρουσιάσουμε παρακάτω, ας δεχθούμε ότι στην τυπική κυψελίδα μας των 100*100 mm^2 είχαμε ταυτόχρονα 2 διαφορετικά μέσα πληρώσεως, το ένα με διηλεκτρική σταθερά 5 και πάχος 2 mm και το άλλο με διηλεκτρική σταθερά 2 και πάχος 3 mm.

Στην περίπτωση αυτή θα πρέπει να εξετάσουμε το πεδίο μέσα σε κάθε υλικό το οποίο παρουσιάζει διαφορετική διηλεκτρική σταθερά και κατά συνέπεια, να βρούμε το δυναμικό στις πλάκες του πυκνωτή του, καθώς και τη συνολική χωρητικότητα του. Στο πρώτο από τα υλικά με διηλεκτρική σταθερά 2 ο τύπος:

$$E = \frac{\sigma}{\varepsilon_0 \varepsilon_\sigma} =$$

μας δίνει μια τιμή η οποία είναι ίση με

$$4 * 10^4 \frac{V}{m}$$

ενώ στο δεύτερο διηλεκτρικό η τιμή είναι

$10^5 \frac{V}{m}$

Άρα το δυναμικό στις πλάκες του πυκνωτή του θα ήταν ίσο με το άθροισμα των 2 δυναμικών, δηλαδή 80 + 300 περίπου 380 V η δε χωρητικότητα θα ήταν ίση με:

$4{,}66*10^{-11}$ Farad.

Από όσα αναφέρονται παραπάνω, συμπεραίνουμε ότι η κυψελίδα μας συμπεριφέρεται σαν ένας πυκνωτής. Η διηλεκτρική σταθερά είναι τα προϊόντα, δηλαδή οι ιστοί μαζί με τους διαλύτες και οι ενώσεις οι οποίες πρόκειται να εκχυλιστούν για να παραληφθούν τα διάφορα μόρια τα οποία μας είναι απαραίτητα.(de Haan & Willcock, 2002; Jain & Rymaszewski, 2004; Kaiser, 1993; Munshi, 1995)

3.2 Επιλογή υλικού για την κατασκευή της κυψελίδας

Είναι προφανές από τα παραπάνω λοιπόν, ότι τα τοιχώματα των κυψελίδων λειτουργούν ως ηλεκτρόδια. Η επιλογή των υλικών για την κατασκευή του ηλεκτροδίου υπάγεται σε μερικούς περιορισμούς. Οι βασικές απαιτήσεις είναι:

- Ηλεκτρικά αγώγιμο
- Υψηλή αντοχή στη διάβρωση (Χημική)
- Αντοχή στην Παραμόρφωση
- Φτηνό
- Μη τοξικό

Σχετικά με τις απαιτήσεις που αναφέρονται παραπάνω, μέταλλα και περιορισμένα πολυμερή μπορούν να επιλέγουν ως κατάλληλα.

Πολυμερή, όπως η πυρρόλη και η πολυανιλίνη είναι παραδείγματα αγώγιμων πολυμερών. Το πολυαιθυλένιο (PE) και το πολυπροπυλένιο (PP) μπορούν επίσης να καταστούν αγώγιμα μετά από υποβολή σε ειδική μεταχείριση. Μέταλλα όπως αλουμίνιο, ανοξείδωτος χάλυβας, χαλκός και το τιτάνιο δύναται επίσης να χρησιμοποιηθούν.

Το αλουμίνιο έχει την υψηλότερη ηλεκτρική αγωγιμότητα μεταξύ των τεσσάρων μετάλλων που προαναφέρονται. Η υψηλή αγωγιμότητα του βοηθά στη μείωση της απώλειας ενέργειας που λαμβάνει χώρα, λόγω της ωμικής θέρμανσης. Ωστόσο, τα μέταλλα βυθιζόμενα σε υγρά τείνουν να διαβρώνονται ευκολότερα. Δενδριτικά νημάτια εμφανίζονται να συνδέουν την άνοδο και την κάθοδο σε πολύ σύντομο χρονικό διάστημα όταν ασκείται παλμικό ηλεκτρικό πεδίο. Αυτό έχει ως αποτέλεσμα τη μείωση

του χρόνου ζωής του ηλεκτροδίου. Επομένως, η επίστρωση με μονωτικά υλικά είναι απαραίτητη για να αποτρέψει τη διάβρωση των μετάλλων.

3.3 Ένταση ηλεκτρικού πεδίου

Η απόσταση διαχωρισμού και η παροχή τάσης καθορίζουν την ισχύ του ηλεκτρικού πεδίου

$$V_2 - V_1 = \int_{p1}^{p2} E * dl$$

Όπου $V_2 - V_1$: Η διαφορά δυναμικού μεταξύ ανόδου P_1 και καθόδου P_2

E το δυναμικό του ηλεκτρικού πεδίου και l η απόσταση μεταξύ των πλακών (ανόδου και καθόδου, πλάκες του πυκνωτή)

Για την απλούστευση μπορούμε να χρησιμοποιήσουμε τον παρακάτω τύπο

$$E = \frac{V}{l}$$

Οπού V η διαφορά δυναμικού μεταξύ ανόδου και καθόδου και l είναι η απόσταση μεταξύ ανόδου και καθόδου.

Από την εξίσωση είναι σαφές ότι είτε αύξηση της τάσης τροφοδοσίας είτε η μείωση της απόστασης μεταξύ ανόδου και καθόδου οδηγεί σε αύξηση του ηλεκτρικού πεδίου. (Ashby & Jones, 2012; Francois Cardarelli, 2008; Simon & Gogotsi, 2008)

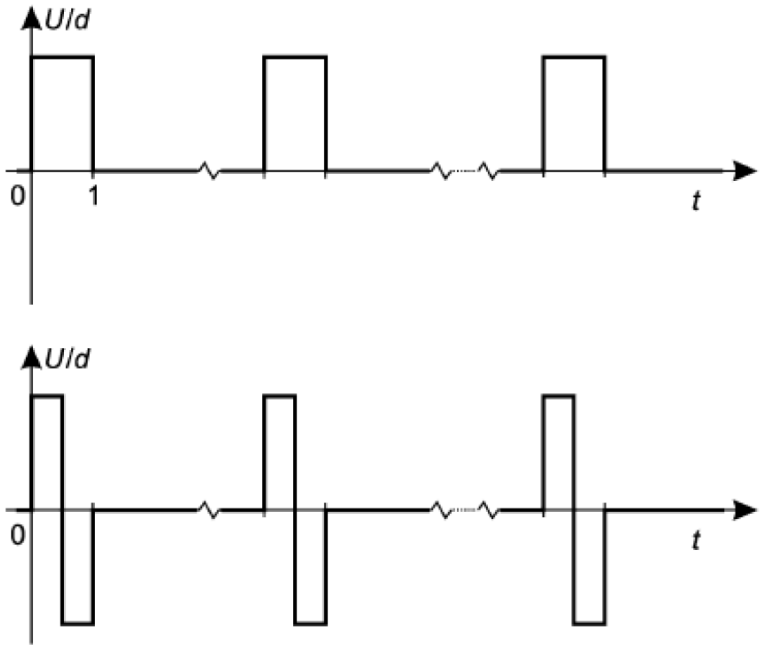
3.4 Η μορφή των παλμών

Υπάρχουν 2 γενικές κατηγορίες παλμών, ο μονοπολικός και ο διπολικός. Αν και ο μονοπολικός παλμός θεωρείται πιο αποτελεσματικός, υπάρχουν ισχυρές ενδείξεις ότι βλάπτει περισσότερο το ηλεκτρόδιο. Το πεδίο Ε που ενεργεί στο ηλεκτρόδιο διαρκώς από την ίδια κατεύθυνση παρέχει πρόσθετη ενέργεια για τα μεταλλικά ιόντα που τείνουν να μεταναστεύσουν από την άνοδο στην κάθοδο.

Ο μονοπολικός παλμός επιλέχθηκε σε αυτό το έργο, καθώς η απόδοση εκχύλισης μπορεί να μεγιστοποιηθεί.

Πέρα από την γενική κατηγορία των παλμών έπρεπε να επιλεχθεί και ο τύπος του παλμού.

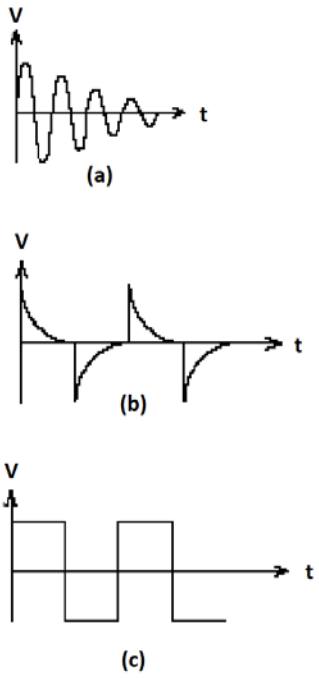
Στην εφαρμογή των ΠΗΠ στα τρόφιμα είτε για λογούς αποστείρωσης είτε για λόγους



Εικόνα 8: Μονοπολικός και Διπολικός παλμός

εκχύλισης, έχουν αναφερθεί τρία είδη παλμών:

- παλμοί ταλάντωσης με αποσύνθεση (ODP- Oscillatory Decay Pulse),
- εκθετικοί παλμοί με αποσύνθεση (EDP- Expontantial Decay Pulse)
- τετραγωνικό κύμα (SW- Square Wave)



Εικόνα 9: Είδη παλμών

ODP αναγνωρίζεται ως ο τύπος παλμού με το μικρότερο επίπεδο αποτελεσματικότητας, γιατί λόγω της φύσης του δεν επιτρέπει τη συνεχή έκθεση σε ηλεκτρικό πεδίο υψηλής έντασης για μεγάλο χρονικό διάστημα. Από την άλλη πλευρά, ενώ το EDP παρουσιάζει μια δραστική αύξηση της τάσης, ο ρυθμός αποσύνθεσης του είναι πολύ αργός με αποτέλεσμα να προκαλεί μια ¨μακριά ουρά¨ που παράγει επιπλέον θερμότητα, η οποία είναι ικανή να καταστρέψει ολοκληρωτικά τα κύτταρα. Ανάμεσα στους τρεις παλμούς, το τετραγωνικό κύμα παρουσιάζει την μέγιστη αποτελεσματικότητα, αφού παραδίδει το μέγιστο δυνατό πεδίο χωρίς να έχει χρόνους αποσύνθεσης. (Griffiths, 2017; Pai & Zhang, 1995)

3.5 Πλάτος παλμού

Ο σχεδιασμός ηλεκτροδίων για το ΠΗΠ είναι στην ουσία ο σχεδιασμός ενός πυκνωτή. Αυτό εξηγήθηκε αναλυτικά στις προηγούμενες ενότητες όπου περιγράψαμε την κυψελίδα σαν ένα πυκνωτή. Σε αυτή την ενότητα, θα ασχοληθούμε με τον χρόνο φόρτισης αυτού του πυκνωτή. Η εξίσωση φόρτισης για τον πυκνωτή μπορεί να εκφραστεί με

$$Q = C * V\left[1 - e^{-\frac{t}{RC}}\right]$$

Όπου Q είναι το συνολικό φορτίο του πυκνωτή, C είναι η χωρητικότητα του πυκνωτή, t είναι ο χρόνος φόρτισης και R η αντίσταση του κυκλώματος

Από την παραπάνω εξίσωση, είναι προφανές ότι ο χρόνος φόρτισης σχετίζεται με

την επιφάνεια του ηλεκτροδίου. Επομένως, εάν η επιφάνεια του ηλεκτροδίου είναι πολύ μεγάλη αλλά το πλάτος του παλμού είναι πολύ μικρό, ο χρόνος φόρτισης του ηλεκτροδίου μπορεί να είναι ανεπαρκής. Έτσι, η ένταση του πεδίου E του συστήματος δεν θα φτάσει την προβλεπόμενη τιμή και η ικανότητα εκχύλισης θα είναι μειωμένη. Ο χρόνος φόρτισης μπορεί να εκφραστεί με R×C. Από αυτό μπορούμε να συμπεράνουμε ότι η κάθε κυψελίδα ανάλογα με τον εμβαδόν της και το υλικό της θα έχει και διαφορετικό ελάχιστο χρόνο φόρτισης.(Barbosa-Cánovas et al., 1999; Barbosa-Cánovas & Altunakar, 2006; Griffiths, 2017).

3.6 Διακόπτες

Ο διακόπτης εκ φόρτισης παίζει επίσης κρίσιμο ρόλο στην απόδοση του συστήματος ΠΗΠ. Ο τύπος του διακόπτη που χρησιμοποιείται θα καθορίσει πόσο γρήγορα μπορεί να αποδώσει και πόσο ρεύμα και διαφορά τάσης μπορεί να αντέξει. Με αυξανόμενη σειρά διάρκειας ζωής, οι κατάλληλοι διακόπτες για συστήματα ΠΗΠ περιλαμβάνουν: ignitrons, spark gap, trigatrons, thyratrons και ημιαγωγούς. Οι διακόπτες ημιαγωγών στερεάς κατάστασης θεωρούνται από τους ειδικούς ως το μέλλον της μεταγωγής υψηλής ισχύος.

Μετά τη συσκευή αποθήκευσης ενέργειας, ο διακόπτης είναι το πιο σημαντικό στοιχείο μιας γεννήτριας παλμών υψηλής ισχύος. Ο διακόπτης ή η συστοιχία είναι τα στοιχεία σύνδεσης μεταξύ της συσκευής αποθήκευσης και του σημείου εκτόνωσης. Ο χρόνος ανόδου, το σχήμα και το πλάτος του παλμού εξόδου της γεννήτριας εξαρτάται σε μεγάλο βαθμό από τις ιδιότητες των διακοπτών. Οι γεννήτριες με χωρητικές συσκευές αποθήκευσης χρειάζονται διακόπτες κλεισίματος, ενώ οι γεννήτριες με επαγωγικές συσκευές αποθήκευσης απαιτούν διακόπτες ανοίγματος (Bluhm 2006).

Υπάρχουν δύο κύριες ομάδες διακοπτών που είναι διαθέσιμες αυτήν τη στιγμή στην αγορά: διακόπτες ON και διακόπτες ON/OFF. Οι διακόπτες ON παρέχουν πλήρη εκ φόρτιση του πυκνωτή, αλλά μπορούν να απενεργοποιηθούν μόνο όταν ολοκληρωθεί η εκ φόρτιση. Οι διακόπτες ON μπορούν να χειριστούν υψηλές τάσεις με σχετικά χαμηλότερο κόστος σε σύγκριση με τους διακόπτες ON/OFF, ωστόσο, η μικρή διάρκεια ζωής και ο χαμηλός ρυθμός επανάληψης είναι μερικά μειονεκτήματα που πρέπει να ληφθούν υπόψη για την επιλογή. Το Ignitron, το Gas Spark Gap, το Trigatron και το Thyratron είναι μερικά από τα παραδείγματα αυτής της ομάδας. Τα τελευταία χρόνια

έχουν αναπτυχθεί διακόπτες τύπου ON/OFF που παρέχουν έλεγχο στη διαδικασία παραγωγής παλμών με μερική ή πλήρη εκ φόρτιση των πυκνωτών (Niayesh & Runde, 2017; Zhang et al., 1995).

3.7 IGBT

Το Insulated Gate Bipolar Transistor που ονομάζεται επίσης IGBT για συντομία, είναι μια διασταύρωση, ένα υβρίδιο μεταξύ ενός συμβατικού Bipolar Junction Transistor (BJT) και ενός Field Effect Transistor (MOSFET) που το καθιστά ιδανικό ως διακόπτη.

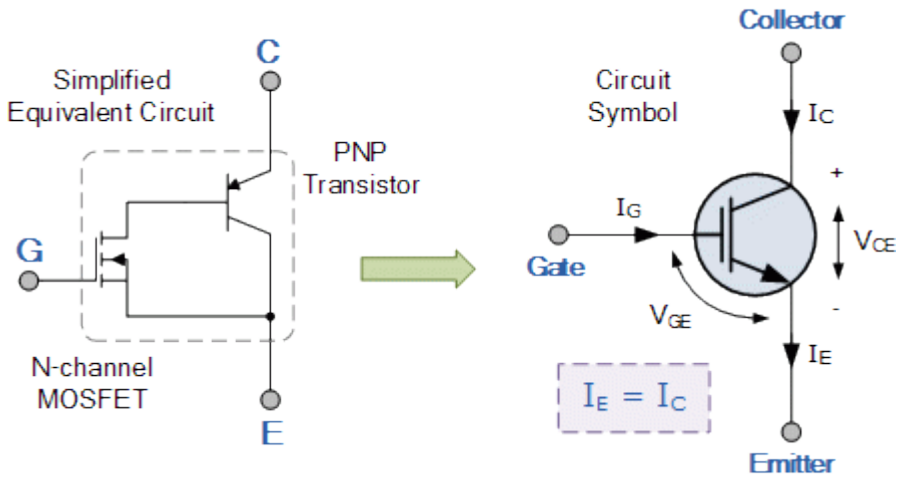
Το τρανζίστορ IGBT παίρνει τα καλύτερα μέρη αυτών των δύο τύπων κοινών τρανζίστορ, την υψηλή σύνθετη αντίσταση εισόδου και τις υψηλές ταχύτητες μεταγωγής ενός MOSFET με τη χαμηλή τάση κορεσμού ενός BJT τρανζίστορ, και τα συνδυάζει μαζί για να παράγει έναν άλλο τύπο τρανζίστορ διακόπτη που είναι ικανό να χειρίζεται μεγάλα ρεύματα συλλέκτη-εκ πομπού με πολύ μικρό ρεύμα για την πύλη εισόδου.

Το Insulated Gate Bipolar Transistor, (IGBT) συνδυάζει την τεχνολογία μονωμένης πύλης (εξ ου και το πρώτο μέρος του ονόματός του) του MOSFET με τα χαρακτηριστικά απόδοσης εξόδου ενός συμβατικού διπολικού τρανζίστορ (εξ ου και το δεύτερο μέρος της ονομασίας του).

Το αποτέλεσμα αυτού του υβριδικού συνδυασμού είναι ότι το «IGBT Transistor» έχει τα χαρακτηριστικά μεταγωγής εξόδου και αγωγιμότητας ενός διπολικού τρανζίστορ, αλλά ελέγχεται από την τάση όπως ένα MOSFET.

Τα IGBT χρησιμοποιούνται κυρίως σε εφαρμογές ηλεκτρονικών που απαιτούν μεγάλη ισχύ, όπως μετατροπείς και τροφοδοτικά, όταν οι απαιτήσεις της συσκευής δεν ικανοποιούνται πλήρως από τα διπολικά τρανζίστορ και τα MOSFET. Υπάρχουν διαθέσιμα διπολικά υψηλού ρεύματος και υψηλής τάσης, αλλά οι ταχύτητες εναλλαγής τους είναι αργές, ενώ τα MOSFET ισχύος μπορεί να έχουν υψηλότερες ταχύτητες εναλλαγής, αλλά οι συσκευές υψηλής τάσης και υψηλού ρεύματος είναι ακριβές και δύσκολο να επιτευχθούν.

Ένα από τα κύρια πλεονεκτήματα του τρανζίστορ IGBT είναι η απλότητα με την οποία μπορεί να ενεργοποιηθεί με την εφαρμογή θετικής τάσης ή να απενεργοποιηθεί κάνοντας το σήμα μηδενικό ή ελαφρώς αρνητικό, επιτρέποντάς του να χρησιμοποιηθεί σε μια ποικιλία εφαρμογών εναλλαγής.



Εικόνα 10:IGBT short circuit

Μπορούμε να δούμε ότι το IGBT είναι μια συσκευή διαγωγιμότητας τριών τερματικών που συνδυάζει μια μονωμένη είσοδο MOSFET N-καναλιών με μια έξοδο διπολικού τρανζίστορ PNP (Positive-Negative-Positive), συνδεδεμένη σε έναν τύπο διαμόρφωσης Darlington.

Ως αποτέλεσμα, τα τερματικά επισημαίνονται ως: Συλλέκτης, Εκπομπός και Πύλη. Δύο από τους ακροδέκτες του (C-E) συνδέονται με τη αγώγιμη διαδρομή από την οποία διέρχεται το ρεύμα, ενώ ο τρίτος ακροδέκτης (G) ελέγχει τη συσκευή.

Όταν χρησιμοποιείται ως στατικός ελεγχόμενος διακόπτης, το IGBT έχει ονομασίες τάσης και ρεύματος παρόμοιες με αυτές του διπολικού τρανζίστορ. Ωστόσο, η παρουσία μιας απομονωμένης πύλης σε ένα IGBT το καθιστά πολύ πιο απλό στην οδήγηση από το BJT, καθώς απαιτείται πολύ λιγότερη ισχύς κίνησης.

Ένα IGBT ενεργοποιείται ή απενεργοποιείται απλώς ελέγχοντας τον ακροδέκτη της πύλης του. Η εφαρμογή ενός θετικού σήματος τάσης εισόδου κατά μήκος της πύλης και του πομπού θα διατηρήσει τη συσκευή στην κατάσταση "ON", ενώ το σήμα της πύλης εισόδου, μηδενικό ή ελαφρώς αρνητικό, θα την κάνει να απενεργοποιηθεί με τον ίδιο τρόπο όπως ένα διπολικό τρανζίστορ ή MOSFET. Ένα άλλο πλεονέκτημα του IGBT είναι ότι έχει πολύ χαμηλότερη αντίσταση καναλιού on-state από ένα τυπικό MOSFET (Chen et al., 2017; Levkov et al., 2019; Rubin et al., 2019).

4. Εφαρμογές των ΠΗΠ στην τεχνολογία τροφίμων

Η τεχνική των ΠΗΠ όπως προαναφέρθηκε είχε απασχολήσει τους επιστήμονες ήδη από τον εικοστό αιώνα. Μια καινοτόμος μέθοδος που επενέβαινε μετακλητά ή αμετάκλητα στη δομή του κυττάρου βρήκε πληθώρα χρήσεων. Οι πρώτες δοκιμές έγιναν στην κατεύθυνση της συντήρησης τροφίμων και την παστερίωσης. Τα θετικά αποτελέσματα έδειξαν ότι η συγκεκριμένη μέθοδος θα μπορούσε να χρησιμοποιηθεί επιτυχώς και αλλού. Ακολουθήσαν δοκιμές για τον συνδυασμό ΠΗΠ και άλλων τεχνικών, έτσι ώστε να επιτευχθεί αφυδάτωση και ψύξη με επίσης θετικά αποτελέσματα. Η τελευταία χρονικά προσπάθεια έγινε στην εκχύλιση βιοδραστικών ενώσεων είτε σαν μοναδική τεχνική είτε σε συνδυασμό με άλλες τεχνικές και αποδείχθηκε ότι μπορεί να απελευθερώσει ενώσεις που αλλιώς θα παρέμεναν εγκλωβισμένες εντός του κυττάρου. Η ένταση του ηλεκτρικού πεδίου, το σχήμα του παλμού, το πλάτος του παλμού, ο χρόνος επεξεργασίας, η συχνότητα και πολικότητα παλμού, η θερμοκρασία, η επεξεργασία σε παρτίδα ή με σύστημα συνεχούς ροής είναι κρίσιμοι παράγοντες που καθορίζουν την αποτελεσματικότητα της τεχνολογίας ΠΗΠ στην επεξεργασία τροφίμων. Η βελτιστοποίηση των παραμέτρων ΠΗΠ είναι ένα απαραίτητο βήμα που απαιτείται για κάθε συγκεκριμένη εφαρμογή.

Πίνακας 2: Ενδεικτικές εφαρμογές της PEF στην τεχνολογία τροφίμων.

Υλικά		Τάση PEF	Επίδραση του PEF	Πήγες
Ξήρανση	Φυλά βασιλικού (Ocimum basilicum)	650 V/cm	Ο χρόνος ξήρανσης μειώθηκε κατά 57% για ξήρανση στον αέρα, 33% για ξήρανση υπό κενό και 25% για ξήρανση με κατάψυξη	Telfser & Gómez Galindo, 2019
	Παστινάκη (Pastinaca sativa)	900 v/cm	Ο χρόνος ξήρανσης μειώθηκε στο 28% στους 70 °C και στο 21% στους 60 °C	(Alam et al., 2018)
εκχύλιση	Πορτοκάλι(Citrus × sinensis)	3000 V/cm	Αύξηση της απόδοσης χυμού κατά 25%	(El Kantar et al., 2018)
	Μύρτιλλο (Vaccinium myrtillus)	5000 V/cm	Αύξηση της απόδοσης του χυμού κατά 28%. Υψηλότερη περιεκτικότητα σε ολικό φαινολικό κατά 43%	(Bobinaitė et al., 2015)

Υλικά		Τάση PEF	Επίδραση του PEF	Πήγες
Ψύξη	Σπανάκι (Spinacia oleracea)	350 V/cm	Βελτίωση της ανοχής στην κατάψυξη	(Demir et al., 2018a)
	Μήλο (Malus domestica)	800 V/cm	Επιτάχυνση των διαδικασιών ψύξης, διατήρηση του σχήματος, αναστολή συρρίκνωσης	(Parniakov et al., 2016a)
Διατήρηση	Μύρτιλλο (Vaccinium myrtillus)	2000 V/cm	Μείωση των *E. coli* και *Listeria innocua* χωρίς αλλαγή του χρώματος και της όψης	(Jin et al., 2017a)
	Γάλα	30000 V/cm	Απενεργοποίηση της αλκαλικής φωσφατάσης. Μειωμένη δραστηριότητα ξανθίνης (30%)	(Sharma et al., 2018)
Αδρανοποίηση σπορίων	*Geobacillus stearothermophilus*	8000 V/cm	Αδρανοποίηση του πληθυσμού κατά 3.28 $\log_{10}$	Reineke et al., 2015
	Bacillus cereus	40000 V/cm	Αδρανοποίηση του πληθυσμού κατά 3.6 $\log_{10}$	Bermúdez-Aguirre et al., 2012
Βιομηχανικά απόβλητα	Κρέας κοτόπουλου	50000 V/cm	Εξαγωγή χρήσιμων μορίων	Ghosh et al., 2019
	Ντομάτα *(Solanum lycopersicum)*	1000 V/cm	Η απόδοση της εκχύλισης καροτενοειδών αυξήθηκε έως και 56,4%	Andreou et al., 2020

4.1 Συντήρηση τροφίμων

Η αλλοίωση των τροφίμων μπορεί να προκληθεί από διάφορους παράγοντες όπως η ανάπτυξη μικροοργανισμών και η δραστηριότητα ενδογενών ενζύμων. Η τεχνολογία ΠΗΠ, σε σύγκριση με τις παραδοσιακές μέθοδούς παστερίωσης, όχι μόνο αδρανοποιεί τους παθογόνους μικροοργανισμούς, αλλά και κάποια ένζυμα, ελαχιστοποιεί την απώλεια της αρχικής γεύσης, του χρώματος, της υφής, των θρεπτικών συστατικών και άλλων θερμοευαίσθητων ενώσεων που βρίσκονται σε τρόφιμα (Abbas Syed, 2017; Vorobiev & Lebovka, 2020). Λόγω των παραπάνω, θεωρείται ένα πολλά υποσχόμενο σύστημα είτε ως πρόσθετη λειτουργία είτε ως υποκατάστατη της παραδοσιακής παστερίωσης με χρήση θερμότητας.
Τα ΠΗΠ μπορεί να χρησιμοποιηθεί με επιτυχία για υγρά προϊόντα με χαμηλό ιξώδες και χαμηλή ηλεκτρική αγωγιμότητα, π.χ. γάλα και χυμούς.

4.2 Μικροβιακή αδρανοποίηση

Είναι γνωστό πως το γάλα και τα γαλακτοκομικά προϊόντα υπόκεινται σε επεξεργασία με διάφορες θερμικές μεθόδους για να καταστούν ασφαλή για ανθρώπινη κατανάλωση. Η λανθασμένη παστερίωση του γάλακτος προκαλεί αλλοίωση του προϊόντος και ανάπτυξη παθογόνων βακτηρίων όπως *Escherichia coli, Listeria spp*. και η *ψευδομονάδα*. Οι διεργασίες κατά τις οποίες χρησιμοποιούνται υψηλές θερμοκρασίες οδηγούν στην απώλεια θρεπτικών συστατικών (Ercolini et al., 2009). Το παλλόμενο ηλεκτρικό πεδίο όχι μόνο αδρανοποιεί τα βακτήρια σε χαμηλές θερμοκρασίες, αλλά επίσης επηρεάζει ελάχιστα τις θρεπτικές και αισθητηριακές ιδιότητες του προϊόντος προς θρέψη. Το ΠΗΠ προκαλεί αδρανοποίηση των Gram-αρνητικών και Gram-θετικών βακτηρίων σε ένα πλήρες γάλα ήδη από τους 50^{o}C (Sharma et al., 2014). Γάλα που έχει υποστεί επεξεργασία θερμικά μπορεί να είναι μικροβιολογικά σταθερό για 21 ημέρες όταν φυλάσσεται στους 4^{o}C. Ωστόσο, η θερμότητα προκαλεί και αρνητικές επιδράσεις όπως: εξασθένηση στις πηκτικές ιδιότητες, μη ενζυματικό μαύρισμα, αποικοδόμηση της λακτόζης, μετουσίωση των πρωτεϊνών κ.α. (Fox et al., 2015). Η τεχνολογία ΠΗΠ μπορεί να χρησιμοποιείται συνεργικά με θερμότητα, αντιμικροβιακούς παράγοντες, φιλτράρισμα μεμβράνης και υπεριώδους ακτινοβολίας προκειμένου να αυξηθεί η αποτελεσματικότητα της βακτηριακής αδρανοποίησης και επιμήκυνση της περιόδου κατανάλωσης.

Οι (Jin et al., 2017b) μελέτησαν πώς το ΠΗΠ επηρεάζει την ιθαγενή μικροχλωρίδα και τον πληθυσμό του *Escherichia coli* και *Listeria innocua*, τα οποία έχουν εμβολιασαν σε βατόμουρα. Ο συνδυασμός ΠΗΠ και PAA (60 ppm Peracetic acid) είχε ως αποτέλεσμα τη μείωση των *Escherichia coli* και *Listeria innocua*, αλλά δεν άλλαξε το χρώμα και την εμφάνιση των βατόμουρων. Το μόνο μειονέκτημα της διαδικασίας ήταν το μαλάκωμα της δομής του μούρου. Στο τελικό εκχυλισμένο δείγμα οι ανθοκυανίνες και φαινολικές ενώσεις αυξήθηκαν κατά 10 και 25% αντίστοιχα, σε σχέση με τα μη επεξεργασμένα δείγματα.

Η έρευνα σχετικά με την εφαρμογή των ΠΗΠ για τον έλεγχο της αλλοίωσης και των παθογόνων μικροοργανισμών σε διαφορετικά προϊόντα αυγών έχει απασχολήσει επίσης έναν ιδιαίτερα μεγάλο αριθμό επιστημόνων. Έχει αναφερθεί ότι το ΠΗΠ μειώνει αποτελεσματικά τη δραστηριότητα διαφόρων μικροοργανισμών σε μια ποικιλία προϊόντων αυγών. Ωστόσο, η επεξεργασία ΠΗΠ αλλάζει επίσης τις δομικές και λειτουργικές ιδιότητες σε κάποιο βαθμό και υπάρχει μεγάλος βαθμός μεταβλητότητας μεταξύ των διαφορετικών μελετών. Ένας από τους κυρίους λογούς που εικάζεται ότι οφείλονται οι μεγάλες διαφορές μεταξύ των αποτελεσμάτων διάφορων ερευνών είναι ότι οι συνθήκες κατά την διάρκεια του ΠΗΠ κατέχουν κεντρικό ρολό και είναι πολύ δύσκολο να επαναληφθούν με ακρίβεια (Monfort et al., 2010; Sampedro et al., 2016; Yogesh, 2016).

4.3 Αδρανοποίηση σπορίων

Το παλλόμενο ηλεκτρικό πεδίο έχει αποδειχθεί ότι είναι αποτελεσματικό και στην αδρανοποίηση σπορίων πέρα των βλαστικών μορφών των κυττάρων όπως αναφέρθηκε παραπάνω. Το ΠΗΠ έχει χρησιμοποιηθεί ως πιθανή εναλλακτική λύση στις παραδοσιακές μεθόδους θερμικής παστερίωσης, καθώς μπορεί να αδρανοποιήσει τα σπόρια διατηρώντας τις αισθητηριακές και θρεπτικές ιδιότητες του τρόφιμου. Το ΠΗΠ έχει αποδειχθεί ότι είναι αποτελεσματικό στη μείωση του πληθυσμού των σπορίων σε μια ποικιλία προϊόντων διατροφής, συμπεριλαμβανομένου του γάλακτος, του χυμού καρότου και του χυμού μήλου (Bermúdez-Aguirre et al., 2012; Choi et al., 2008; Raso et al., 1998; Reineke et al., 2015; Spilimbergo et al., 2003). Παρόλο που η χρήση του ΠΗΠ σε κάποιες περιπτώσεις επιτυγχάνει ικανοποιητικά αποτελέσματα σε σχέση με την αδρανοποίηση σπορίων εν τούτης ο συνδυασμός του με άλλες μεθόδους μπορεί να τα βελτιστοποιήσει. Παραδείγματος χάρη, ο συνδυασμός του ΠΗΠ εντός ενός συστήματος με υψηλή πίεση μπορεί να επιτύχει υψηλοτέρα επίπεδα αδρανοποίησης σπορών σε σχέση με μια απλή θερμική επεξεργασία (Choi et al., 2008).

4.4 Ξήρανση

Μεταξύ των μη θερμικών τεχνολογιών που χρησιμοποιούνται ως προ επεξεργασία για την ενίσχυση της διαδικασίας ξήρανσης, το παλλόμενο ηλεκτρικό πεδίο φαίνεται να παρουσιάζει τις μεγαλύτερες δυνατότητες. Η εμπορική επιτυχία της εφαρμογής του ΠΗΠ στην επεξεργασία πατάτας δείχνει ότι είναι δυνατό να χρησιμοποιηθεί ως υποστήριξη για παραδοσιακές μεθόδους σε βιομηχανική κλίμακα (Lebovka et al., 2007; Wu & Zhang, 2014). Η υπάρχουσα βιβλιογραφία παρέχει στοιχεία που αποδεικνύουν ότι λόγω του φαινομένου της ηλεκτροδιάτρησης, η επεξεργασία με ΠΗΠ διαρρηγνύει την κυτταρική δομή και έτσι εντείνει την απομάκρυνση του νερού στις διαδικασίες ξήρανσης. Η τροποποίηση της δομής του κυττάρου επηρεάζει τον χρόνο και τον τρόπο κατάψυξης. Αντίστοιχα επηρεάζει και τον τρόπο που η λυοφιλοποίηση λαμβάνει χώρα. Ο μικρότερος χρόνος ξήρανσης ή λυοφιλίωσης συνεπάγεται επίσης ποιοτικές αλλαγές στο προεπεξεργασμένο υλικό. Ωστόσο, οι αλλαγές στις φυσικές και χημικές ιδιότητες των προκατεργασμένων αποξηραμένων τροφίμων με την χρήση του ΠΗΠ εξαρτώνται από πολλούς παράγοντες που σχετίζονται τόσο με το προϊόν όσο και με τη διαδικασία. Έτσι, η θετική επίδραση του ΠΗΠ τόσο στην ποιότητα όσο και στην κινητική μπορεί να απαιτεί προσαρμογή των παραμέτρων ξήρανσης (Punthi et al., 2022; Toepfl and Knorr, 2008; Wiktor et al., 2022). Η χρήση του παλλόμενου ηλεκτρικού πεδίου σε συνδυασμό με την ξήρανση έχει και θετικό αντίκρισμα στα θρεπτικά και οργανοληπτικά χαρακτηριστικά των τροφίμων πέρα από την εξοικονόμηση χρόνου και ενέργειας. Για παράδειγμα, έχει

διαπιστωθεί ότι η επεξεργασία με ΠΗΠ ακολουθούμενη από ξήρανση σε κενό βελτίωσε την ποιότητα και τη διάρκεια ζωής των αποξηραμένων φετών μήλου (Chauhan et al., 2018; Matys et al., 2022; Parniakov et al., 2016b). Ακόμα η επεξεργασία με ΠΗΠ ακολουθούμενη από ξήρανση βελτίωσε σημαντικά το χρώμα και την υφή των αποξηραμένων βατόμουρων. Οι μελέτες διαπίστωσαν ότι η επεξεργασία με ΠΗΠ είχε ως αποτέλεσμα την υψηλότερη αντιοξειδωτική δράση και την υψηλότερη περιεκτικότητα σε σάκχαρα στα αποξηραμένα βατόμουρα (Yu et al., Jin, & Xiao, 2017; Yu, Jin, Fan, et al., 2017). Αυτές οι μελέτες καταδεικνύουν τη δυνατότητα του ΠΗΠ ως τεχνικής συντήρησης για τη βελτίωση της ποιότητας και της διάρκειας ζωής των αποξηραμένων τροφίμων και ποτών. Η επεξεργασία με ΠΗΠ πριν από την ξήρανση μπορεί να βοηθήσει στην αδρανοποίηση μικροοργανισμών, ενζύμων και στη διατήρηση της θρεπτικής αξίας και της αισθητηριακής ποιότητας του τελικού προϊόντος.

4.5 Ψύξη

Η κατάψυξη των τροφίμων αντιμετωπίζει ένα σημαντικό πρόβλημα, τον σχηματισμό κρυστάλλων πάγου που μπορούν να καταστρέψουν τον ιστό. έτσι ώστε μετά την απόψυξη τα προϊόντα (για παράδειγμα μαλακά φρούτα, φυλλώδη λαχανικά) να χάνουν το σχήμα τους και να παραμένουν νωπά. Σε αυτήν τη μορφή, δεν βρίσκουν μεγάλη απήχηση στο καταναλωτικό κοινό. Έχει αποδειχθεί ότι το παλλόμενο ηλεκτρικό πεδίο μπορεί να χρησιμοποιηθεί για τη βελτίωση της ανοχής στο πάγωμα διάφορων φύλλων. Το ΠΗΠ έχει εφαρμοστεί σε συνδυασμό με εμποτισμό υπό κενό παρουσία κρυοπροστατευτικών όπως η τρεαλόζη, η σακχαρόζη, η γλυκόζη και η φρουκτόζη. Ο συνδυασμός αυτών των μεθόδων είχε ως αποτέλεσμα τα κύτταρα των φύλλων να παραμείνουν βιώσιμα (viable) και τα φύλλα να διατηρήσουν τη στρεβλότητα μετά τον κύκλο κατάψυξης και απόψυξης (Ammelt et al., 2021; Demir et al., 2018b; Shayanfar et al., 2014). Επίσης, έρευνες έχουν δείξει ότι ανάλογα με τον τρόπο που εφαρμόζεται η ψύξη στα τρόφιμα, τα αποτελέσματα του ΠΗΠ μπορεί να διαφέρουν. Συγκεκριμένα, όταν ο χρόνος ψύξης είναι πολύ μικρός, τότε η επίδραση του ΠΗΠ τόσο στην ποιότητα του τελικού προϊόντος όσο και στον χρόνο ψύξης είναι πολύ σημαντική. Όσο ο χρόνος ψύξης αυξάνεται τόσο παρατηρείται ότι τα τελικά προϊόντα, επεξεργασμένα με ΠΗΠ και μη, έχουν κοινά χαρακτηριστικά (Ammar et al., 2009; Nowak & Jakubczyk, 2022). Συμπεραίνουμε λοιπόν, ότι το ΠΗΠ σε συνδυασμό με διάφορους μεθόδους ψύξης μπορεί να μειώσει τον χρόνο και την ενέργεια που απαιτεί η διαδικασία, καθώς και να προστατεύσει τα οργανοληπτικά χαρακτηριστικά των τροφών.

4.6 Εκχύλιση βιοδραστικών ενώσεων

Η εκχύλιση ως τρόπος απόκτησης βιοδραστικών ενώσεων είναι μια από τις πιο συχνά χρησιμοποιούμενες διαδικασίες στη βιομηχανία. Συνήθως, η διαδικασία της εκχύλισης περιλαμβάνει χημική ή/και θερμική επεξεργασία ενός δείγματος. Πολυάριθμες μελέτες αναφέρουν ότι η εφαρμογή παλλόμενου ηλεκτρικού πεδίου μπορεί να ενισχύσει την αποτελεσματικότητά της διαδικασίας, να μειώσει τον χρόνο εκχύλισης και να ελαχιστοποιήσει τυχόν ζημιές στις εξαγόμενες ενώσεις. Το ΠΗΠ έχει χρησιμοποιηθεί για τη βελτίωση της εκχύλισης ενδοκυτταρικών ενώσεων από φρούτα και λαχανικά. Έρευνες έχουν δείξει ότι η υποβοηθούμενη εκχύλιση με παλλόμενο ηλεκτρικό πεδίο μπορεί να αυξήσει την εκχυλισιμότητα πολυφαινολών που προέρχονται από διάφορα φρούτα και λαχανικά (Luengo et al., 2013; Ntourtoglou, et al., 2022; Segovia et al., 2015). Επιπλέον η χρήση παλλομένου ηλεκτρικού πεδίου πριν ή και κατά τη διάρκεια της εκχύλισης μπορεί να μειώσει αισθητά τον χρόνο της διαδικασίας (Liu et al., 2019; Ntourtoglou et al., 2020; Silve et al., 2018). Η ικανότητα του ΠΗΠ να αδρανοποιεί τους μικροοργανισμούς και να προκαλεί τη διαπερατότητα των ευκαρυωτικών κυττάρων χωρίς σημαντική αύξηση της θερμοκρασία του προϊόντος μπορεί να χρησιμοποιηθεί στη διαδικασία της παραγωγής κρασιού για τη βελτίωση της ποιότητάς του. Η χαμηλή κατανάλωση ενέργειας και ο σύντομος χρόνος επεξεργασίας που απαιτείται για τη διαπερατότητα των κυττάρων του φλοιού των σταφυλιών είναι τα βασικά πλεονεκτήματα της χρήσης του ΠΗΠ στην απόκτηση κρασιών με υψηλή περιεκτικότητα σε φαινολικές ενώσεις. Η υψηλή συγκέντρωση πολυφαινολών βοηθά στη σταθεροποίηση του χρώματος και βελτιώνει την ποιότητα του κρασιού κατά τη διαδικασία της παλαίωσης (Boulton, 2001). Οι φαινολικές ενώσεις έχουν επίσης δράσεις υπέρ της υγείας όπως αντιοξειδωτικές και αντιφλεγμονώδεις ιδιότητες. Το ΠΗΠ μπορεί να επηρεάσει τη γεύση, το χρώμα ή τη θρεπτική αξία του γλεύκους σταφυλιών και του κρασιού. Το ΠΗΠ δύναται να μειώσει επίσης την ποσότητα SO_2, που μπορεί να επηρεάσει την ποιότητα του κρασιού (Corrales et al., 2008; Drosou et al., 2017b; Puértolas et al., 2010). Η χρήση, λοιπόν, του ΠΗΠ στην ενίσχυση της διαδικασίας της εκχύλισης είτε αυτοτελώς είτε σε συνδυασμό με άλλες μεθόδους, αυξάνει τις περισσότερες φορές τις παραληφθείσες βιοδραστικές ουσίες με ταυτόχρονη μείωση του χρόνου και του ενεργειακού αποτυπώματος.

4.7 Τροποποίηση του αμύλου

Το παλλόμενο ηλεκτρικό πεδίο μπορεί να χρησιμοποιηθεί για την τροποποίηση του αμύλου της πατάτας, του καλαμποκιού, του σιταριού, του ρυζιού και άλλων σιτηρών, οσπρίων και καρπών. Οι ερευνητές έχουν παρατηρήσει μείωση της θερμοκρασίας ζελατινοποίησης, λόγω της αναδιάταξης και της καταστροφής της μοριακής δομής του αμύλου μετά την επεξεργασία με ΠΗΠ. Η εφαρμογή του ΠΗΠ επηρεάζει την πέψη του αμύλου καθώς αυξάνει τα επίπεδα του ταχέως εύπεπτου αμύλου στην πατάτα, σιτάρι, στα μπιζελιά κ.α. Επίσης μέθοδοι τροποποίησης του αμύλου όπως η ακετυλίωση μπορούν να ενισχυθούν σημαντικά με την επεξεργασία με ΠΗΠ. Η χρήση του ΠΗΠ για την βοήθεια των διαδικασιών στην τροποποίηση του αμύλου μπορεί να βελτιώσει την αποτελεσματικότητα της διαδικασίας, να μειώσει τον χρόνο αντίδρασης και να εξοικονόμηση αντιδραστήρια (Han et al., 2009; Hong et al., 2018; Q. Li et al., 2019; Zeng et al., 2016).

4.8 Αξιοποίηση αποβλήτων από βιομηχανία τροφίμων και ποτών

Η βιομηχανία τροφίμων παράγει τεράστιες ποσότητες υποπροϊόντων και απόβλητων, τα οποία δημιουργούν έντονα προβλήματα, καθώς η απόρριψη τους σχετίζεται με θέματα περιβάλλοντος και υγείας. Από την άλλη πλευρά, αυτά τα απορρίμματα πλούσια σε φυσικές βιοδραστικες ενώσεις, ιδιαίτερα αυτά που προέρχονται από τη βιομηχανία φρούτων και λαχανικών. Τα τελευταία χρόνια, δίνεται ιδιαίτερη έμφαση στην βιωσιμότητα των τροπών παραγωγής, με αποτέλεσμα “πράσινες” διαδικασίες όπως το ΠΗΠ να χρησιμοποιούνται για τη μείωση του όγκου των αποβλήτων και για την ανάκτηση ενώσεων από αυτά. Για παράδειγμα, οι Ghosh et al., 2019 πρότειναν τον συνδυασμό ΠΗΠ με μηχανική πίεση για την εξαγωγή χρήσιμων χημικών ενώσεων από τα απόβλητα του στήθους κοτόπουλου. Αντίστοιχα, οι Andreou et al., 2020 εφάρμοσαν την τεχνική του ΠΗΠ σε διαφορά στάδια της επεξεργασία τομάτας για τη βέλτιστη αξιοποίηση των αποβλήτων της. Ακόμη, η χρήση του ΠΗΠ ως στάδιο προ επεξεργασίας μπορεί να ενισχύσει σημαντικά την εκχύλιση πολυφαινολικών ενώσεων από τα στελέχη σταφυλιών που συνήθως αντιμετωπίζονται ως απόβλητα μετά το τέλος της οινοποίησης (Ntourtoglou, et al., 2022). Συμπεραίνουμε λοιπόν ότι χρήση του ΠΗΠ μπορεί είτε να δημιουργήσει είτε να ενισχύσει ένα σύστημα ανατροφοδότησης σημαντικών ενώσεων στη βιομηχανία τροφίμων, χρησιμοποιώντας ως πρώτη ύλη τα βιομηχανικά απόβλητα.

5. Παλλόμενο ηλεκτρικό πεδίο σε συνδυασμό με άλλες μηχανικές μεθόδους

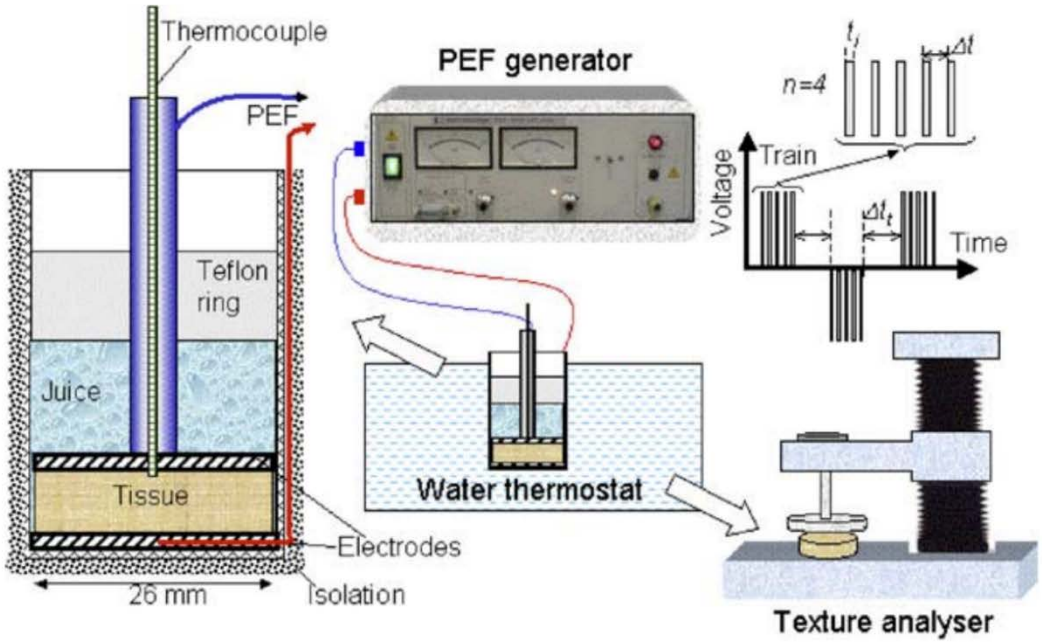
Σήμερα, το παλλόμενο ηλεκτρικό πεδίο έχει αποδειχθεί ότι αποτελεί μια μη θερμική επεξεργασία τροφίμων με αντιμικροβιακά χαρακτηριστικά και με την ικανότητα να βελτιώνει την εκχυλισιμότητα διάφορων πρώτων υλών σε θερμοκρασία περιβάλλοντος. Σύμφωνα με την τεχνολογία εμποδίων (hurdle concept) (Leistner, 2000), ο συνδυασμός ΠΗΠ με άλλες μεθόδους συντήρησης μπορεί να μειώσει τον χρόνο και την ενέργεια, αλλά και να ενισχύσει την πιθανότητα ένα κύτταρο να οδηγηθεί στον θάνατο (Leistner, 2000). Η επίδραση του ΠΗΠ με την προσθήκη αντιμικροβιακών ενώσεων φυσικής προέλευσης, όπως η νισίνη, έχει λάβει ιδιαίτερη προσοχή, αν και άλλες αναδυόμενες μη θερμικές τεχνικές, όπως π.χ. η χρήση διοξειδίου του άνθρακα υψηλής πίεσης, μπορούν επίσης να ληφθούν υπόψη (Pataro et al., 2010a; Pol et al., 2000). Επιπλέον, η βακτηριοκτόνος δράση που προκύπτει από την ταυτόχρονη εφαρμογή ΠΗΠ και συμβατικών θερμικών επεξεργασιών υποδηλώνουν τη δυνατότητα μείωσης της ένταση της θέρμανσης διατηρώντας παράλληλα τη μικροβιακή αποδοχή. Ακόμα, η χρήση του ΠΗΠ μαζί με μικροκύματα αποτελεί έναν πολύ αποτελεσματικός συνδυασμός σε καταστάσεις στις οποίες μεμονωμένα δεν θα μπορούσαν να έχουν τα επιθυμητά αποτελέσματα. Επίσης, ο συνδυασμός των προηγουμένων μπορεί να φέρει σύνθετα αποτελέσματα, όπως η χρήση του ΠΗΠ ως τεχνολογία αποσύνθεσης κυττάρων και οι υπέρηχοι ως μέσο για την επίδραση στα φαινόμενα διεπαφής (Manzoor et al., 2019a; Martín-Belloso & Sobrino-López, 2011; Ntourtoglou, et al., 2022; Ostermeier et al., 2021a).

Πίνακας 3: Παλλόμενο ηλεκτρικό πεδίο σε συνδυασμό με άλλες μηχανικές μεθόδους

Συνδυασμός διεργασιών	Συνθήκες	Μικροοργανισμός	Λογαριθμική μείωση	Μέσο	Πηγή
PEF + υπέρηχοι	12 kV/cm + 20 kHz	*Saccharomyces cerevisiae*	3.7	Κρασί ρυζιού	(Lyu et al., 2016)
	40 kV/cm + 30 kHz	*Staphylococcus aureus*	6,8	Χυμός πορτοκαλιού	(Walkling-Ribeiro, et al., 2009)
PEF+HPCD	6 kV/cm+8.0 MPa	*Saccharomyces cerevisiae*	7,7	Ρυθμιστικό διάλυμα	(Pataro et al., 2010b)
	25 kV/cm+20 MPa	*B. cereus*	4,0	Διάλυμα γλυκερόλης	(Spilimbergo et al., 2003b)
PEF+UV	40 kV/cm+30 w	*S. aureus*	9,5	Χυμός μήλου	(Walkling-Ribeiro, et al., 2009)
	60 kV/cm+25w	*E. coli*	5.35	Χυμός μήλου	(Gachovska et al., 2008)
PEF + ηπία θερμοκρασία	34 kV/cm+55 °C	*E. coli*	7,0	Smoothie τροπικών φρούτων	(Walkling-Ribeiro, et al., 2009)
	43 kV/cm+55 °C	*Salmonella typhimurium*	5,9	Χυμός μήλου	(El-Hag et al., 2010)
PEF και Υψηλή Υδροστατική Πίεση	30 kV/cm+ 400 MPa	*Listeria innocua*	4	Νερό	(Pyatkovskyy et al., 2018a)
	12 kV/cm+700 MPa	*Bacillus Subtilis*	7.1	Ρυθμιστικό διάλυμα+ Χυμός πορτοκαλιού	(Sasagawa et al., 2006)

5.1 ΠΗΠ και Ήπια Θερμοκρασία

Η αύξηση της θερμοκρασίας προκαλεί απώλεια της ελαστικότητας της μεμβράνης και μείωση του πάχους της λιπιδικής διπλοστιβάδας, λόγω της μετάβασης των φωσφολιπίδιων της μεμβράνης από γέλη σε υγρό-κρυσταλλική δομή, έτσι ώστε τα κύτταρα να είναι πιο ευαίσθητα στα ΠΗΠ (Garcia-Manyes et al., 2005; Kučerka et al., 2011; Williamson et al., 1975). Επίσης, η ηλεκτροδιάτρηση της μεμβράνης επηρεάζεται από τη θερμοκρασία και η θερμοκρασία μπορεί να επηρεάσει την επίδραση του ΠΗΠ στον φυτικό ιστό (Zimmermann, 1986). Όπως είναι αναμενόμενο, το ΠΗΠ σε συνδυασμό με μια θερμική επεξεργασία αυξάνει την πιθανότητα του βακτηριακού θανάτου (Fox et al., 2008). Οι χρήσεις του ΠΗΠ σε συνδυασμό με ήπια θέρμανση μπορεί να προσεγγιστούν με δύο διαφορετικούς τρόπους: (1) εφαρμογή των ΠΗΠ πριν ή μετά από μέτρια θέρμανση του τρόφιμου και (2) έλεγχος της θερμοκρασίας κατά την επεξεργασία ΠΗΠ. Έρευνες έχουν δείξει ότι η αύξηση της θερμοκρασίας στους 50°C πριν αλλά και κατά την διάρκεια του ΠΗΠ οδηγεί σε σημαντική αύξηση της απενεργοποίησης του *Saccharomyces cerevisiae* (Katiyo et al., 2017; Montanari et al., 2019).

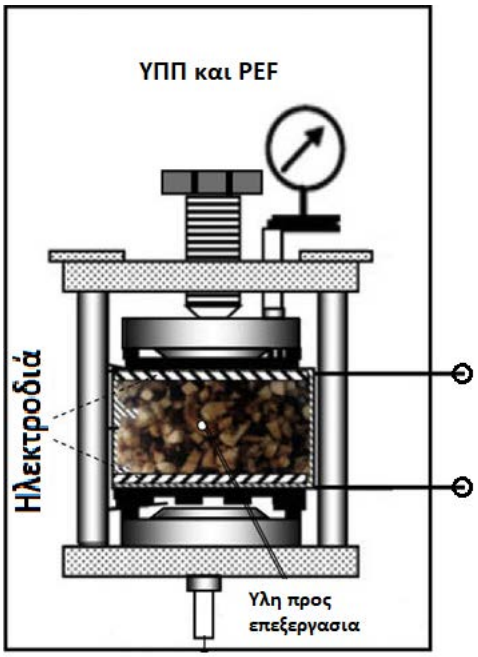


Εικόνα 11: Γεννήτρια παλλομένων ηλεκτρικών πεδίων σε συνδυασμό με τεχνητή αύξηση της θερμοκρασίας (Lebovka et al., 2005)

5.2 ΠΗΠ και Υπέρηχοι

Είναι γνωστό ότι οι υπέρηχοι έχουν χρησιμοποιηθεί στο παρελθόν για τον καθαρισμό εργαστηριακού και βιομηχανικού εξοπλισμού, όπως επίσης και για την απενεργοποίηση συγκεκριμένων μικροοργανισμών (Furuta et al., 2004; Islam et al., 2013; Mason, 2016; Mawson et al., 2011). Μια από τις θετικές προοπτικές της εκχύλισης με την χρήση ΠΗΠ είναι ότι έχουμε την ικανότητα να αποκτάμε πολύτιμα συστατικά που μέχρι πρότινος μπορούσαμε να τα αποκτήσουμε μόνο με θερμικούς τρόπους επεξεργασίας (Meglič et al., 2021; Sitzmann et al., 2017). Η υποβοηθούμενη από υπερήχους εκχύλιση έχει την τάση να αυξάνει τον ρυθμό και την έκταση με την οποία η μάζα μεταφέρεται, λόγω των μηχανικών επιδράσεών της, ενισχύοντας τη διείσδυση του διαλύτη στο εσωτερικό του κυττάρου μέσω της διάσπασης των κυτταρικών τοιχωμάτων (Rhazi et al., 2019). Αυτές οι δυο τεχνικές έχουν θετικό αντίκτυπο στην απόδοση και την ποιότητα της εκχύλισης με χρήση όσο το δυνατόν λιγότερων οργανικών διαλυτών, καθώς και μείωση των ενεργειακών δαπανών και του χρόνου παραγωγής. Επίσης, ενισχύουν τη θρεπτική αξία των προϊόντων διατροφής, λόγω της ελάχιστης θερμικής επεξεργασίας των ευαίσθητων στη θερμότητα θρεπτικών ουσιών. Ο συνδυασμός των τεχνικών ΠΗΠ και των υπέρηχων έχουν τραβήξει το ενδιαφέρον διάφορων επιστημόνων, ερευνητών αλλά και επενδυτών, λόγω της οικονομίας, της απλότητας, της σταθερότητας και της αποτελεσματικότητάς τους για την απόκτηση βιοδραστικών συστατικών (Li et al., 2021; Manzoor et al., 2019b; Ntourtoglou, et al., 2022). Επίσης, άξιο αναφοράς είναι ότι ο συνδυασμός αυτών των δυο τεχνικών μπορεί να επηρεάσει και δευτερογενείς μεθόδους επεξεργασίας, όπως το τηγάνισμα, αφού επηρεάζει ποιοτικές παραμέτρους, όπως η υγρασία, το λίπος και η περιεκτικότητα σε ακρυλαμίδιο (Ostermeier et al., 2021b). Τέλος, αξίζει να αναφέρουμε ότι χωρίς την κοινή χρήση των προαναφερόμενων τεχνικών, καμία από τις δυο δεν θα μπορούσε να έχει τα βέλτιστα επιθυμητά αποτελέσματα. Από μηχανολογικής πλευράς, δεν έχει κατασκευαστεί κάποια εγκατάσταση που να εμπεριέχει και τις δυο τεχνικές σε ένα κοινό όργανο.

5.3 ΠΗΠ και Υψηλή Υδροστατική Πίεση



Εικόνα 12: ΥΠΠ και ΠΗΠ

Η επεξεργασία με Υψηλή Υδροστατική Πίεση (ΥΥΠ) μπορεί να πραγματοποιηθεί είτε κατά παρτίδες είτε σε ημισυνεχή λειτουργία. Σε μια διαδικασία παρτίδας, το προϊόν θα τοποθετηθεί ελεύθερο είτε μέσα σε ατομικές ειδικά διαμορφωμένες συσκευασίες. Σε ένα ημισυνεχές σύστημα, το προϊόν τοποθετείται απευθείας σε έναν θάλαμο επεξεργασίας και χωρίζεται από το μέσο πίεσης με ένα πλωτό έμβολο. Εγκαθιστώντας έναν αριθμό από μονάδες πίεσης παράλληλα, η ημισυνεχής λειτουργία μπορεί να παρέχει συνεχή ροή προϊόντος σε ασηπτικές εγκαταστάσεις συσκευασίας (Leadley, 2008). Δοχεία με ανθεκτικά τοιχώματα και με ενσωματωμένα ηλεκτρόδια μπορούν να κατασκευαστούν έτσι ώστε το ΠΗΠ να μπορεί να λάβει χώρα ταυτόχρονα με ΥΥΠ (Pyatkovskyy et al., 2018b). Ο λόγος της ιδίας φύσης της ΥΥΠ είναι ότι συνήθως χρησιμοποιείται για παστερίωση και όχι για ενίσχυση της εκχύλισης. Η αποτελεσματικότητα της επεξεργασίας με ΠΗΠ επηρεάζεται σε μεγάλο βαθμό από την εφαρμοζόμενη ένταση ηλεκτρικού πεδίου με υψηλότερο ρυθμό αδρανοποίησης σε υψηλότερη τάση. Οι διαδοχικά εφαρμοσμένες θεραπείες ΥΥΠ και ΠΗΠ έδειξαν ως επί το πλείστον αθροιστικά αποτελέσματα. Ωστόσο, παρατηρήθηκαν συνεργατικά αποτελέσματα όταν η ΥΥΠ και το ΠΗΠ εφαρμόστηκαν ταυτόχρονα. Η ταυτόχρονη επεξεργασία με ΥΥΠ- ΠΗΠ οδηγεί σε μέγιστη αλλαγή στο δυναμικό ζήτα, υποδηλώνοντας μεγαλύτερη διαρροή ενδοκυτταρικών συστατικών (Pyatkovskyy et al., 2018b; Sasagawa et al., 2006; Xia et al., 2022).

5.4 ΠΗΠ και Υψηλής Πίεσης Διοξείδιο Του Άνθρακα

Η επεξεργασία υψηλής πίεσης διοξειδίου του άνθρακα (ΥΠΔΑ-HPCD-high pressure carbon dioxide) συνεπάγεται την εφαρμογή διοξειδίου άνθρακα υπό πίεση (CO_2) για ορισμένο χρόνο είτε σε παρτίδα, είτε σε ημι-παρτίδα είτε σε συνεχή λειτουργία για την απενεργοποίηση μικροοργανισμών (McIntyre & McNeil, 1997; Tomasula et al., 1997). Εξαιτίας των λιποφιλικών και υδροφιλικών ιδιοτήτων του, το CO_2 διαχέεται μέσω και μέσα στην κυτταρική μεμβράνη, αυξάνοντας τη ρευστότητά της, εξάγοντας τα ζωτικά συστατικά της και διαταράσσοντας το βιολογικό της σύστημα (Garcia-Gonzalez et al., 2007) . Η ηλεκτροδιάτρηση μπορεί να ενισχύσει τη μεταφορά μάζας του υπό πίεση CO_2 μέσα στο κύτταρο και επομένως να επιταχύνει την κυτταρική κατάρρευση (Garcia-Gonzalez et al., 2007). Λόγω της φύση της διεργασίας με ΥΠΔΑ, ο κυρίαρχος ρόλος για τον οποίο μπορεί να χρησιμοποιηθεί σε συνδυασμό με το ΠΗΠ είναι για παστερίωση ή για μερική μείωση πληθυσμού βακτηρίων. Στον συνδυασμό των δυο παραπάνω μεθόδων, το ΠΗΠ οφείλει να προηγείται της ΥΠΔΑ, λόγω του ότι ο σκοπός είναι να επιτευχθεί ηλεκτροδιαπερατότητα, έτσι ώστε το CO_2 να μπορεί να εισέλθει με μεγαλύτερη ευκολία στο εσωτερικό του κυττάρου (Pataro et al., 2010b; Spilimbergo et al., 2003).

5.5 ΠΗΠ και Υπεριώδης Ακτινοβολία

Η υπεριώδης ακτινοβολία (Ultraviolet-UV) οφείλει την βακτηριοκτόνο δράση της στις φωτοχημικές αντιδράσεις και ελεύθερες ρίζες που σχηματίζονται πάνω από τα επιφανειακά στρώματα των ακτινοβολημένων τροφίμων (Ibarz et al., 2005). Ο συνδυασμός των δυο μεθόδων έχει προσθετικά αποτελέσματα στη μείωση του πληθυσμού των βακτηρίων. Η σειρά με την οποία μπορούν να εφαρμοστούν οι παραπάνω τεχνολογίες δεν φαίνεται να επηρεάζει το τελικό αποτέλεσμα (Gachovska et al., 2008). Επίσης, ο συνδυασμός των δυο μεθόδων διατήρησε ποιοτικά χαρακτηριστικά όπως χρώμα και υφή, τα οποία με τη χρήση υψηλής θερμοκρασίας θα είχαν χαθεί (Noci et al., 2008).

6. Περιλήψεις δημοσιεύσεων

6.1 Δημοσίευση Ι

Pulsed Electric Field Extraction of α and β-Acids from Pellets of *Humulus lupulus* (Hop)

Σε αυτήν την εργασία διερευνήθηκαν η διαδικασία της εκχύλισης λυκίσκου χρησιμοποιώντας την τεχνική του παλλόμενου ηλεκτρικού πεδίου, καθώς και οι διαφορετικές επιδράσεις της σε δυο ποικιλίες λυκίσκου, μία πικρή και μια αρωματική. Η επίδραση του PEF στην εκχύλιση αξιολογήθηκε με μέτρηση της συγκέντρωσης α-οξέων και β-οξέων (χουμουλόνες και λουπουλόνες). Όσον αφορά τον αρωματικό χαρακτήρα, το πτητικό καρυοφυλλένιο, το χουμουλένιο και το β-μυρσένιο του λυκίσκου αναλύθηκαν τόσο με όσο και χωρίς τη χρήση της επεξεργασίας PEF. Για την ανάλυση των οξέων και του πτητικού κλάσματος εφαρμόστηκε η αναλυτική μέθοδος της φασματοφωτομετρίας UV-Vis ακολουθούμενη από αέρια χρωματογραφία σε συνδυασμό με φασματομετρία μάζας. Για τη δεύτερη τεχνική, τα εκχυλίσματα είχαν προηγουμένως καθαριστεί μέσω σύριγγας Graphitized Black Carbon για Εκχύλιση Στερεάς Φάσης. Τα αποτελέσματα αποκάλυψαν ότι το PEF είχε θετική επίδραση στα α-οξέα του πικρικού λυκίσκου αυξάνοντας τον ρυθμό εκχύλισης αυτών των οξέων κατά 20%, ενώ τα πτητικά παρουσίασαν αύξηση 5,6 και 7,4% για το χουμουλένιο και το καρυοφυλλένιο, αντίστοιχα. Όσον αφορά την αρωματική ποικιλία του λυκίσκου, η επεξεργασία PEF δεν φάνηκε να έχει αξιοσημείωτα αποτελέσματα στις συνθήκες που εφαρμόστηκαν.

6.2 Δημοσίευση II

In situ Creation of the Natural Phenolic Aromas of Beer: A Pulsed Electric Field Applied to Wort-Enriched Flax Seeds

Για τη λεπτομερή παραγωγή φαινολικών αρωμάτων στην μπύρα, εφαρμόστηκε ένα παλλόμενο ηλεκτρικό πεδίο στο γλεύκος μπύρας, το οποίο ήταν εμπλουτισμένο με σπόρους λιναριού. Η επιλογή των σπόρων λιναριού ως πηγής φερουλικού οξέος βασίστηκε στην υψηλή περιεκτικότητά τους σε πρόδρομες ουσίες φερουλικού και στην εγγενή θρεπτική τους αξία. Το PEF εφαρμόστηκε σε αλεσμένους σπόρους λιναριού, με και χωρίς βήτα γλυκοσιδάση. Η ζύμωση πραγματοποιήθηκε με στελέχη ζύμης *Saccharomyces* και μη *Saccharomyces*.. Επιπλέον, η 4-βινυλγουαϊακόλη (4-VG), ένα μόριο με εξαιρετικά έντονο άρωμα (πτητική φαινόλη), παρήχθη με αποκαρβοξυλίωση του φερουλικού οξέος ή του προδρόμου του και αδρανούς στη γεύση (4-υδροξυ-3-μεθοξυκινναμικό οξύ). Όλα τα στελέχη ζύμης μπορέσαν να μεταβολίσουν το φαιρουλικό οξύ σε 4-VG, χρησιμοποιώντας την καθαρή ένωση μέσα σε ένα συνθετικό μέσο ή την ύπαρξη της σε σπόρους λιναριού, με την μεγαλύτερη απόδοση να εντοπίζεται από τη ζύμωση με *Saccharomyces cerevisiae* ως πρόδρομο. Η μέθοδος είχε απόδοση παραγωγής 4-VG έως και 120%. Οι πειραματικές συνθήκες διεξήχθησαν με E= 1 kV/cm, συνολικός χρόνος επεξεργασίας 15 λεπτά (χρόνος πεδίου ti = 1 μs, χρόνος παύσης tp = 1 ms, Συνολικοί παλμοί 9003). Η αποτελεσματικότητα της εφαρμογής ήταν ανεξάρτητη από αυτή της ζύμης.

6.3 Δημοσίευση III

Use of Pulsed Electric Field as a Low-Temperature and High-Performance "Green" Extraction Technique for the Recovery of High Added Value Compounds from Olive Leaves

Τα φύλλα ελιάς, ένα υποπροϊόν των γεωργικών αποβλήτων, θεωρούνται σημαντική βιολογική πηγή πολυφαινολών, γνωστές ως βιοδραστικές ενώσεις. Σε αυτήν την μελέτη αξιολογήθηκε η τεχνική του παλλόμενου ηλεκτρικού πεδίου για την εξαγωγή πολυφαινολών από φύλλα ελιάς. Οι παράμετροι της μελέτης περιλάμβαναν μια σειρά ¨πράσινων¨ διαλυτών (αιθανόλη, νερό, καθώς και μείγματα αυτών σε αναλογίες 25%, 50%, 75%) και διαφορετικές τιμές για τη διάρκεια παλμού του PEF. Ο βαθμός εκχύλισης αξιολογήθηκε χρησιμοποιώντας αναλύσεις συγκέντρωσης ολικής φαινόλης (μέθοδος Folin–Ciocalteu) και υγρής χρωματογραφίας υψηλής απόδοσης (HPLC), ενώ η αντιοξειδωτική δράση αξιολογήθηκε χρησιμοποιώντας θερμιδομετρία διαφορικής σάρωσης. Τα αποτελέσματα που ελήφθησαν από τα εκχυλίσματα PEF συγκρίθηκαν με αυτά των εκχυλισμάτων που παράγονται χωρίς την εφαρμογή PEF. Το υψηλότερο αποτέλεσμα PEF παρατηρήθηκε για υδατική αιθανόλη, 25% v/v, χρησιμοποιώντας διάρκεια παλμού 10 μs. Η αύξηση των συνολικών πολυφαινολών έφτασε το 31,85%, ενώ η αύξηση στους συγκεκριμένους μεταβολίτες έφτασε το 265,67%. Η ανάκτηση στις πολυφαινόλες βρέθηκε ότι εξαρτάται από τον διαλύτη, τη διάρκεια παλμού της διεργασίας και τη δομή των μεταβολιτών που εκχυλίστηκαν.

6.4 Δημοσίευση IV

Optimization of Pulsed Electric Field as Standalone “Green” Extraction Procedure for the Recovery of High Value-Added Compounds from Fresh Olive Leaves

Στη συνέχεια της προηγουμένης έρευνας αξιολογήθηκε η αξιοποίηση των αποβλήτων φύλλων ελιάς μέσω της μεγιστοποίησης της συγκέντρωσης πολυφαινόλης στα εκχυλίσματα. Ερευνήθηκαν επίσης περισσότερες παράμετροι για τησυνεισφορά της PEF στην ~~στατική~~ εκχύλιση διαλείποντος έργου στερεού-υγρού των φύλλων ελιάς επιλέγοντας και ρυθμίζοντας με ακρίβεια σημαντικές παραμέτρους του PEF, όπως η γεωμετρία του θαλάμου εκχύλισης, η ένταση του ηλεκτρικού πεδίου, η διάρκεια παλμού, η περίοδος παλμού (και η συχνότητα) και η διάρκεια εκχύλισης. Τα παραχθέντα εκχυλίσματα αξιολογήθηκαν μέσω σύγκρισης μεταξύ τους και έναντι εκχυλισμάτων που ελήφθησαν χωρίς την εφαρμογή PEF. Για τον προσδιορισμό της απόδοσης εκχύλισης χρησιμοποιήθηκαν η μέθοδος Folin-Ciocalteu, η υγρή χρωματογραφία υψηλής απόδοσης και η διαφορική θερμιδομετρία σάρωσης. Η βέλτιστη συνεισφορά του PEF στην ικανότητα εκχύλισης ολικών πολυφαινολών (αύξηση 38% με αύξηση 117% για συγκεκριμένους μεταβολίτες) παρουσιάστηκε για ορθογώνιο θάλαμο εκχύλισης, 25% v/v αιθανόλη:νερού, διάρκεια παλμού (tpulse) 2 μs, ένταση ηλεκτρικού πεδίου (E) 0,85 kV/cm, περίοδος 100 μs (T) και διάρκεια εκχύλισης 15 λεπτών (εκχύλιση), επιβεβαιώνοντας μια σημαντική εξάρτηση της απόδοσης της εκχύλισης με το PEF από τις επιλεγμένες παραμέτρους.

6.5 Δημοσίευση V

Extraction of volatile aroma compounds from toasted oak wood using pulsed electric field

Σε αυτήν την έρευνα προσδιορίστηκε η επίδραση του παλλόμενου ηλεκτρικού πεδίου στην εκχύλιση πτητικών ενώσεων από ψημένα ροκανίδια βελανιδιάς βυθισμένα σε διάφορα υδατικά διαλύματα αιθανόλης (5%, 12%, 50% και 70% v/v). Ως μέθοδος ανάλυσης επιλέχτηκε η αέρια χρωματογραφία σε συνδυασμό με φασματοσκοπία μάζας και η εκχύλιση ήταν υποβοηθουμένη από υπερήχους. Η επεξεργασία με PEF έδειξε την υψηλότερη επίδραση στο διάλυμα 5%, αυξάνοντας τη βανιλίνη, τη συριγγαλδεΰδη, τη λακτόνη δρυός (*Cis* and *trans* ισομερή της β-methyl-γ-οκταλακτόνης) και τη φουρφουράλη κατά 75%, 371%, 13% και 50%, αντίστοιχα. Το PEF δοκιμάστηκε επίσης σε Αγιωργίτικο κόκκινο κρασί, σε βύνη και αποστάγματα κρασιού. Για το κόκκινο κρασί, το PEF με ισχύ 1,2 kV/cm αύξησε την απόδοση της εκχύλισης των ενώσεων του αρωματικού ξύλου από 5% σε 200%. Διαφορές στις συγκεντρώσεις των εκχυλισμένων πτητικών ενώσεων μεταξύ του μάρτυρα και των δειγμάτων που υποβλήθηκαν σε επεξεργασία με PEF παρατηρήθηκαν επίσης στα αποστάγματα βύνης και κρασιού. Η οργανοληπτική αξιολόγηση έδειξε ότι η βύνη που έχει υποστεί επεξεργασία με PEF ήταν παρόμοια με ένα παλαιωμένο ουίσκι, με αποχρώσεις φρυγανισμένης βελανιδιάς.

6.6 Δημοσίευση VI

Pulsed Electric Field and *Salvia officinalis* L. Leaves: A Successful Combination for the Extraction of High Value Added Compounds

Η παρούσα μελέτη είχε ως στόχο να αξιολογήσει την υποβοηθούμενη από παλλόμενο ηλεκτρικό πεδίο εκχύλιση φυτοχημικών από τα φύλλα του *Salvia officinalis L.* Οι παράμετροι που μελετήθηκαν περιλάμβαναν μια διάρκεια παλμού 10 και 100 μs για 30 λεπτά, χρησιμοποιώντας διαφορετικούς ¨πράσινους¨ διαλύτες: καθαρή αιθανόλη, καθαρό νερό και τα μείγματά τους σε συγκεντρώσεις 25, 50 και 75% v/v. Τα εκχυλίσματα που προέκυψαν αξιολογήθηκαν έναντι εκχυλισμάτων αναφοράς που ελήφθησαν χωρίς PEF. Για την εκτίμηση της αποτελεσματικότητας της εκχύλισης, προσδιορίστηκε η περιεκτικότητα σε ολικές πολυφαινόλες, μεμονωμένες πολυφαινόλες και πτητικές ενώσεις, καθώς και η αντοχή στην οξείδωση. Η βέλτιστη συνεισφορά του PEF στις ολικές και μεμονωμένες πολυφαινόλες, ροσμαρινικό οξύ, ικανότητα εκχύλισης (έως 73,2% και 403,1% αύξηση, αντίστοιχα) λήφθηκε στον υδατικό διαλύτη αιθανόλης 25%v/v χρησιμοποιώντας διάρκεια παλμού 100 μs. Το PEF αποδείχθηκε ότι επηρεάζει επίσης την τελική συγκέντρωση και τη σύνθεση των πτητικών ενώσεων των εκχυλισμάτων που λαμβάνονται.

6.7 Δημοσίευση VII

Hyphenated Extraction of Valuable Compounds from *Aesculus carnea*: Ultrasound Extraction with Pulsed Electric Field Pretreatment

Σε αυτήν την εργασία ασχοληθήκαμε με τα υποπροϊόντα της υλοτόμησης και του κλαδέματος. Η εξαγωγή πολύτιμων ενώσεων από φυσικά υποπροϊόντα είναι μια σύγχρονη τάση που βοηθάει στην ελαχιστοποίηση του περιβαντολογικού αποτυπώματος. Η χρήση του παλλόμενου ηλεκτρικού πεδίου αξιολογήθηκε ως στάδιο προεπεξεργασίας, πριν από την υποβοηθούμενη από υπερήχους εκχύλιση φαινολικών ενώσεων από φύλλα *Aesculus carnea*. Επιπλέον, εξετάστηκαν διάφορες συγκεντρώσεις διαλυτών, καθώς και ο χρόνος προεπεξεργασίας με PEF. Σύμφωνα με τα αποτελέσματα, έως και 33% περισσότερες φαινολικές ενώσεις μπορούν να εκχυλιστούν, υπό βέλτιστες συνθήκες (30% αιθανόλη σε νερό ως διαλύτης και προεπεξεργασία PEF για 30 λεπτά, σε σύγκριση με τον ίδιο διαλύτη, χωρίς PEF). Επιπλέον, ο χρόνος επεξεργασίας με PEF δεν έδειξε διαφορετικά αποτελέσματα και δεν καταγράφηκαν διαφορές, υποδηλώνοντας ότι χαμηλότερος χρόνος επεξεργασίας μπορεί να αποφέρει την ίδια εκχύλιση φαινολικών ενώσεων. Ως εκ τούτου, η χρήση του PEF συνιστάται ιδιαίτερα σε συνδυασμό με εκχύλιση με υπερήχους, για τη μεγιστοποίηση της απόδοσης των φαινολικών ενώσεων που εξάγονται από τα φύλλα του *Aesculus carnea*.

6.8 Δημοσίευση VIII

Combination of Pulsed Electric Field and Ultrasound in the Extraction of Polyphenols and Volatile Compounds from Grape Stems.

Είναι γνωστό οι βόστρυχοι των σταφυλιών αποτελούν υποπροϊόν κατά τη διαδικασία της οινοποίησης, παρά την υψηλή περιεκτικότητά τους σε πολλές πολύτιμες ενώσεις. Ο στόχος αυτής της εργασίας ήταν να εξεταστεί εάν η χρήση παλλόμενου ηλεκτρικού πεδίου θα μπορούσε να αυξήσει την απόδοση πολυφαινόλης και πτητικών ενώσεων στα εκχυλίσματα. Για τον λόγο αυτό, χρησιμοποιήθηκε μια σχετικά χαμηλής κατανάλωσης ενέργειας διεργασία PEF (χαμηλή ένταση ηλεκτρικού πεδίου, 1 kV/cm) για μικρό χρονικό διάστημα (30 λεπτά) στους βόστρυχες των σταφυλιών. Επιπλέον, εξετάστηκε η επίδραση διαφορετικών διαλυτών κατά τη διάρκεια του σταδίου της προεπεξεργασίας. Με τη χρήση της ανάλυσης Folin–Ciocalteu, τα εκχυλίσματα συγκρίθηκαν με τα αντίστοιχα δείγματα ελέγχου (όχι προεπεξεργασμένα με PEF). Επιπλέον, παρασκευάστηκαν εκχυλίσματα για να αξιολογηθεί εάν συμβαίνουν αλλαγές στο πτητικό προφίλ των εκχυλισμάτων. Τα αποτελέσματα έδειξαν ότι όχι μόνο το PEF μπορεί να αυξήσει την απόδοση των πολυφαινολών (καταγράφηκε αύξηση έως και 35%), αλλά και ότι ο διαλύτης που χρησιμοποιείται κατά την προεπεξεργασία του PEF μπορεί να επηρεάσει τη διαδικασία. Επιπλέον, καταγράφηκε αύξηση 234% στη συνολική περιεκτικότητα σε πτητικές ενώσεις, όταν το PEF χρησιμοποιήθηκε ως στάδιο προεπεξεργασίας. Επομένως, ο συνδυασμός PEF και εκχύλισης με τη βοήθεια υπερήχων είναι πολλά υποσχόμενος για τη λήψη βιοδραστικών ενώσεων από βόστρυχες σταφυλιού.

6.9 Δημοσίευση IX

Pulsed electric field: A “green” extraction technology for biomolecular products from glycerol with fermentation of non-Saccharomyces yeasts

Η γλυκερόλη είναι το κύριο οργανικό υποπροϊόν της βιομηχανίας βιοντίζελ και είναι πηγή άνθρακα για ζυμώσεις ή υπόστρωμα για βιομετασχηματισμούς. Αυτή η εργασία διερεύνησε αν το παλλόμενο ηλεκτρικό πεδίο μπορεί να χρησιμοποιηθεί για να ενισχύσει την εκχύλιση πολυόλης και προπανοδιόλης από ζυμώσεις με βάση γλυκερίνη/γλυκόζη. Μελετήθηκαν τρία διαφορετικά εμπορικά, μη *Saccharomyces* στελέχη, το *Torulaspora delbrueckii* Prelude (Hansen), το *Torulaspora delbrueckii* Biodiva 291 (Lallemand) και το *Metschnikowia pulcherrima* (Lallemand). Τα αποτελέσματα έδειξαν ότι το PEF είχε θετικό αντίκτυπο στην εκχύλιση πολυολών που κυμαίνεται από 12 έως 191%, ανεξάρτητα από τις συνθήκες ζύμωσης. Το στέλεχος *Torulaspora delbrueckii* Biodiva 291 (Lalemand) βρέθηκε ότι είναι πιο αποτελεσματικό σε pH 7,1. Αξιολογήθηκε μια βελτιστοποιημένη μέθοδος με βάση την αέρια χρωματογραφία για τον ποιοτικό και ποσοτικό προσδιορισμό των σχηματισμένων προϊόντων. Τα πειράματα πραγματοποιήθηκαν είτε σε φιάλες είτε σε βιοαντιδραστήρα.

7. Συμπεράσματα

Για αυτήν τη διατριβή κατασκευάστηκε ένα σύστημα παλλόμενου ηλεκτρικού πεδίου έχοντας λάβει υπόψη τα υπάρχοντα εμπορικά συστήματα τόσο εργαστηριακής, όσο και βιομηχανικής κλίμακας. Χαρακτηριστική διαφορά του προκείμενου συστήματος είναι η προσθήκη ενός διακόπτη IGBT και η λειτουργία του με ελάχιστη κατανάλωση ενέργειας. Επίσης, κατασκευάστηκαν ηλεκτρόδια με τα οποία αποδείχθηκε ότι ανάλογα με τον τύπο της εκχύλισης ή τη φύση του υποστρώματος ή εν γένει το υλικό προς επεξεργασία, διαφέρουν ως προς την αποτελεσματικότητα τους.

Η διατριβή αυτή συνεισέφερεστην τεκμηρίωση ότι η ηλεκτροδιαπερατότητα που προκαλεί η τεχνική του παλλομένου ηλεκτρικού πεδίου στα μικροβιακά, φυτικά ή και ζωικά κύτταρα είναι δυνατόν να χρησιμοποιηθεί για την εκχύλιση βιο ενεργών, ενώσεων, φαρμακευτικού, διατροφικού ή απλώς τεχνικού ενδιαφέροντος, όπως αρωματικές ενώσεις ή παράγωγα βιο-μετατροπών. Με την τεχνική του PEF που χρησιμοποιήθηκε επιβεβαιώθηκε ότι πέρα από διαδικασίες διάσπασης του κυττάρου, όπως η παστερίωση, η τεχνική μπορεί να συμβάλει στην βελτιστοποίηση διαδικασιών εκχύλισης. Με τη χρήση της τεχνικής μπορούν να απομονωθούν σημαντικές ουσίες, μειώνοντας κατά πολύ το ενεργειακό και οικονομικό κόστος. Τέλος, αποδείχθηκε ότι τόσο το PEF μόνο του όσο και σε συνδυασμό με άλλες μεθόδους είναι ικανό να μειώσει τον απαραίτητο χρόνο μιας διαδικασίας σε σύγκριση με τις κλασικές μεθόδους.

Προφανώς, δεν κατέστη δυνατή η μελέτη όλων τους ενώσεων οι οποίες μπορεί να επηρεάζονταν από την χρήση του PEF σε μια διαδικασία. Έτσι, επικεντρωθήκαμε σε αρωματικές ενώσεις που συναντώνται στα ποτά και παράγονται με διαδικασίες εκχύλισης είτε από το περιβάλλον τους είτε με προσθήκες στο εσωτερικό τους. Στη συνέχεια, τεκμηριώσαμε ότι σημαντικές ουσίες για την ανθρώπινη υγεία, όπως οι τανίνες, οι ανθοκυανίνες και τα φαινολικά είναι δυνατόν να ανακτηθούν από γεωργικά απόβλητα, όπως οι βόστρυχοι ή τα φύλλα ελιάς, με τη βοήθεια του PEF.

Εν κατακλείδι, θα μπορούσαμε να συνοψίσουμε ότι με τη διατριβή αυτή αποδεικνύεται ότι η τεχνική του PEF είναι μια νέα τεχνική απομόνωσης και παραλαβής σημαντικών ενώσεων από φυτικούς ιστούς, μικροβιακές ζυμώσεις, ακόμα και από υποστρώματα πιο περίπλοκα μέσα σε ποικίλα περιβάλλοντα όπως υδατοαλκοολικά διαλύματα, μείγματα μεθανόλης νερού και υγρά ζυμώσεων που περιλαμβάνουν ζωντανά μικροβιακά κύτταρα. Με τον τρόπο αυτό δείχνουμε ότι η τεχνική αυτή μπορεί να θεωρηθεί ότι είναι μια πράσινη τεχνική, φιλική προς το περιβάλλον, χωρίς να απαιτεί τη χρήση επικίνδυνων διαλυτών ή θερμικών επεξεργασιών οι οποίες μπορούν να καταστρέψουν το προϊόν και έχουν μεγάλο ενεργειακό αποτύπωμα.

Ταυτόχρονα με την παραπάνω έρευνα, αποδείξαμε ότι η τεχνική του PEF μπορεί να εφαρμοστεί με έναν καινοτόμο τεχνολογικά εξοπλισμό, πιο απλό, χωρίς να απαιτούνται ιδιαίτερα ηλεκτρικά πεδία και συνεπώς τεχνικά λιγότερο απαιτητικό. Ανοίχτηκε έτσι ο δρόμος για να μπορεί να χρησιμοποιηθεί από περισσότερα εργαστήρια και για περισσότερες έρευνες.

Ο εξοπλισμός που κατασκευάστηκε εμπεριέχει κυκλώματα που μπορούν να παράγουν ηλεκτρικούς παλμούς από 1000V έως 5000V και διάφορων σχημάτων και μεγεθών ηλεκτρόδια. Το σχεδιασμένο ηλεκτρόδιο κατέχει καθοριστικό ρολό στην αποτελεσματικότητα της εκχύλισης κατά την εκτέλεση της διαδικασίας. Επομένως, η επιλογή υλικού και ο σχεδιασμός είναι κομβικής σημασίας για τη συνολική πορεία. Για λόγους σταθερότητας και ηλεκτρικής αγωγιμότητας χρησιμοποιήθηκε το ανοξείδωτο ατσάλι και ο χαλκός.

8. ΒΙΒΛΙΟΓΡΑΦΙΑ

Abbas Syed, Q. (2017). Pulsed Electric Field Technology in Food Preservation: A Review. *Journal of Nutritional Health & Food Engineering*, *6*(6). https://doi.org/10.15406/JNHFE.2017.06.00219

Alam, M. R., Lyng, J. G., Frontuto, D., Marra, F., & Cinquanta, L. (2018). Effect of Pulsed Electric Field Pretreatment on Drying Kinetics, Color, and Texture of Parsnip and Carrot. *Journal of Food Science*, *83*(8), 2159–2166. https://doi.org/10.1111/1750-3841.14216

Ammar, J., Hecke, E., Lebovka, N., Vorobiev, E., & Lanoisellé, J.-L. (2009). *Pulsed electric fields improve freezing process*. 95–100.

Ammelt, D., Lammerskitten, A., Wiktor, A., Barba, F. J., Toepfl, S., & Parniakov, O. (2021). The impact of pulsed electric fields on quality parameters of freeze-dried red beets and pineapples. *International Journal of Food Science & Technology*, *56*(4), 1777–1787. https://doi.org/10.1111/IJFS.14803

Andreou, V., Dimopoulos, G., Dermesonlouoglou, E., & Taoukis, P. (2020). Application of pulsed electric fields to improve product yield and waste valorization in industrial tomato processing. *Journal of Food Engineering*, *270*, 109778. https://doi.org/10.1016/J.JFOODENG.2019.109778

Ashby, M. M., & Jones, D. R. H. (2012). Engineering Materials 1. *Engineering Materials 1*. https://doi.org/10.1016/C2009-0-64288-4

Barbosa-Cánovas, G. v., & Altunakar, B. (2006). Pulsed Electric Fields Processing of Foods: An Overview. *Food Engineering Series*, 3–26. https://doi.org/10.1007/978-0-387-31122-7_1

Barbosa-Cánovas, G. v., Góngora-Nieto, M. M., Pothakamury, U. R., & Swanson, B. G. (1999). Fundamentals of High-Intensity Pulsed Electric Fields (PEF). *Preservation of Foods with Pulsed Electric Fields*, 1–19. https://doi.org/10.1016/B978-012078149-2/50002-7

Bermúdez-Aguirre, D., Dunne, C. P., & Barbosa-Cánovas, G. V. (2012). Effect of processing parameters on inactivation of Bacillus cereus spores in milk using

pulsed electric fields. *International Dairy Journal*, *24*(1), 13–21. https://doi.org/10.1016/J.IDAIRYJ.2011.11.003

Bobinaitė, R., Pataro, G., Lamanauskas, N., Šatkauskas, S., Viškelis, P., & Ferrari, G. (2015). Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products. *Journal of Food Science and Technology*, *52*(9), 5898. https://doi.org/10.1007/S13197-014-1668-0

Boulton, R. (2001). The Copigmentation of Anthocyanins and Its Role in the Color of Red Wine: A Critical Review. *American Journal of Enology and Viticulture*, *52*(2), 67–87. https://doi.org/10.5344/AJEV.2001.52.2.67

Carter, M., & Shieh, J. (2015). Gene Delivery Strategies. *Guide to Research Techniques in Neuroscience*, 239–252. https://doi.org/10.1016/B978-0-12-800511-8.00011-3

Chauhan, O. P., Sayanfar, S., & Toepfl, S. (2018). Effect of pulsed electric field on texture and drying time of apple slices. *Journal of Food Science and Technology*, *55*(6), 2251. https://doi.org/10.1007/S13197-018-3142-X

Chen, X., Yu, L., Jiang, T., Tian, H., Huang, K., & Wang, J. (2017). A High-Voltage Solid-State Switch Based on Series Connection of IGBTs for PEF Applications. *IEEE Transactions on Plasma Science*, *45*(8), 2328–2334. https://doi.org/10.1109/TPS.2017.2713781

Choi, J., Wang, D., Namihira, T., Katsuki, S., Akiyama, H., Lin, X., Sato, H., Seta, H., Matsubara, H., & Saeki, T. (2008). Inactivation of spores using pulsed electric field in a pressurized flow system. *Journal of Applied Physics*, *104*(9), 094701. https://doi.org/10.1063/1.3006440

Corrales, M., Toepfl, S., Butz, P., Knorr, D., & Tauscher, D. (2008). Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison. *Innovative Food Science and Emerging Technologies*, 85–91.

De Haan, S. W. H., & Willcock, P. R. (2002). Comparison of the energy performance of pulse generation circuits for PEF. *Innovative Food Science & Emerging Technologies*, *3*(4), 349–356. https://doi.org/10.1016/S1466-8564(02)00069-3

Demir, E., Dymek, K., & Galindo, F. G. (2018a). Technology Allowing Baby Spinach Leaves to Acquire Freezing Tolerance. *Food and Bioprocess Technology*, *11*(4), 809–817. https://doi.org/10.1007/S11947-017-2044-7

Drosou, F., Yang, E., Marinea, M., Dourtoglou, E. G., Chatzilazarou, A., & Dourtoglou, V. G. (2017a). An assessment of potential applications with pulsed electric field in wines. *BIO Web of Conferences*, *9*, 02010. https://doi.org/10.1051/BIOCONF/20170902010

El Kantar, S., Boussetta, N., Lebovka, N., Foucart, F., Rajha, H. N., Maroun, R. G., Louka, N., & Vorobiev, E. (2018). Pulsed electric field treatment of citrus fruits: Improvement of juice and polyphenols extraction. *Innovative Food Science & Emerging Technologies*, *46*, 153–161. https://doi.org/10.1016/J.IFSET.2017.09.024

El-Hag, A. H., Dadarwal, R., Gonzalez, O. R., Jayaram, S. H., & Griffiths, M. W. (2010). Survivability of inoculated versus naturally grown bacteria in apple juice under pulsed electric fields. *IEEE Transactions on Industry Applications*, *46*(1), 9–15. https://doi.org/10.1109/TIA.2009.2036513

Ercolini, D., Russo, F., Ferrocino, I., & Villani, F. (2009). Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology*, *26*(2), 228–231. https://doi.org/10.1016/J.FM.2008.09.005

Fox, M. B., Esveld, D. C., Mastwijk, H., & Boom, R. M. (2008). Inactivation of L. plantarum in a PEF microreactor: The effect of pulse width and temperature on the inactivation. *Innovative Food Science & Emerging Technologies*, *9*(1), 101–108. https://doi.org/10.1016/J.IFSET.2007.06.007

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). Dairy chemistry and biochemistry, second edition. *Dairy Chemistry and Biochemistry, Second Edition*, 1–584. https://doi.org/10.1007/978-3-319-14892-2/COVER

Francois Cardarelli. (2008). Materials Handbook. *Materials Handbook*. https://doi.org/10.1007/978-1-84628-669-8

Furuta, M., Yamaguchi, M., Tsukamoto, T., Yim, B., Stavarache, C. E., Hasiba, K., & Maeda, Y. (2004). Inactivation of Escherichia coli by ultrasonic irradiation. *Ultrasonics Sonochemistry*, *11*(2), 57–60. https://doi.org/10.1016/S1350-4177(03)00136-6

Gachovska, T. K., Kumar, S., Thippareddi, H., Subbiah, J., & Williams, F. (2008). Ultraviolet and pulsed electric field treatments have additive effect on inactivation of E. coli in apple juice. *Journal of Food Science*, *73*(9). https://doi.org/10.1111/J.1750-3841.2008.00956.X

Garcia-Gonzalez, L., Geeraerd, A. H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Van Impe, J. F., & Devlieghere, F. (2007). High pressure carbon dioxide inactivation of microorganisms in foods: the past, the present and the

future. *International Journal of Food Microbiology*, *117*(1), 1–28. https://doi.org/10.1016/J.IJFOODMICRO.2007.02.018

Garcia-Manyes, S., Oncins, G., & Sanz, F. (2005). Effect of Temperature on the Nanomechanics of Lipid Bilayers Studied by Force Spectroscopy. *Biophysical Journal*, *89*(6), 4261. https://doi.org/10.1529/BIOPHYSJ.105.065581

Ghosh, S., Gillis, A., Sheviryov, J., Levkov, K., & Golberg, A. (2019). Towards waste meat biorefinery: Extraction of proteins from waste chicken meat with non-thermal pulsed electric fields and mechanical pressing. *Journal of Cleaner Production*, *208*, 220–231. https://doi.org/10.1016/J.JCLEPRO.2018.10.037

Gimsa, J., & Wachner, D. (2001). Analytical Description of the Transmembrane Voltage Induced on Arbitrarily Oriented Ellipsoidal and Cylindrical Cells. *Biophysical Journal*, *81*(4), 1888–1896. https://doi.org/10.1016/S0006-3495(01)75840-7

Golberg, A., & Rubinsky, B. (2013). Mass Transfer Phenomena in Electroporation. In *Transport in Biological Media* (pp. 455–492). Elsevier Inc. https://doi.org/10.1016/B978-0-12-415824-5.00012-6

Griffiths, D. J. (2017). Introduction to Electrodynamics. *Introduction to Electrodynamics*. https://doi.org/10.1017/9781108333511

Han, Z., Zeng, X. an, Zhang, B. shan, & Yu, S. juan. (2009). Effects of pulsed electric fields (PEF) treatment on the properties of corn starch. *Journal of Food Engineering*, *93*(3), 318–323. https://doi.org/10.1016/J.JFOODENG.2009.01.040

HEINZ, D. (1960). *Verfahren und Vorrichtung zur Gewinnung der einzelnen Phasen aus dispersen Systemen* (Issue DE 1237541 B). https://lens.org/075-367-470-683-646

Hong, J., Zeng, X. A., Han, Z., & Brennan, C. S. (2018). Effect of pulsed electric fields treatment on the nanostructure of esterified potato starch and their potential glycemic digestibility. *Innovative Food Science & Emerging Technologies*, *45*, 438–446. https://doi.org/10.1016/J.IFSET.2017.11.009

Ibarz, A., Pagán, J., Panadés, R., & Garza, S. (2005). Photochemical destruction of color compounds in fruit juices. *Journal of Food Engineering*, *69*(2), 155–160. https://doi.org/10.1016/J.JFOODENG.2004.08.006

Islam, M. N., Zhang, M., & Adhikari, B. (2013). The Inactivation of Enzymes by Ultrasound—A Review of Potential Mechanisms. *Http://Dx.Doi.Org/10.1080/87559129.2013.853772*, *30*(1), 1–21. https://doi.org/10.1080/87559129.2013.853772

Jain, P., & Rymaszewski, E. J. (2004). Thin-Film Capacitors for Packaged Electronics. *Thin-Film Capacitors for Packaged Electronics*. https://doi.org/10.1007/978-1-4419-9144-7

Jin, T. Z., Yu, Y., & Gurtler, J. B. (2017a). Effects of pulsed electric field processing on microbial survival, quality change and nutritional characteristics of blueberries. *LWT*, *77*, 517–524. https://doi.org/10.1016/J.LWT.2016.12.009

Joardder, M. U. H., & Masud, M. H. (2019). A Brief History of Food Preservation. In *Food Preservation in Developing Countries: Challenges and Solutions* (pp. 57–66). Springer International Publishing. https://doi.org/10.1007/978-3-030-11530-2_3

Kaiser, C. J. (1993). The Capacitor Handbook. *The Capacitor Handbook*. https://doi.org/10.1007/978-94-011-8090-0

Katiyo, W., Yang, R., & Zhao, W. (2017). Effects of combined pulsed electric fields and mild temperature pasteurization on microbial inactivation and physicochemical properties of cloudy red apple juice (Malus pumila Niedzwetzkyana (Dieck)). *Journal of Food Safety*, *37*(4), e12369. https://doi.org/10.1111/JFS.12369

Kotnik, T., & Miklavčič, D. (2000). Analytical Description of Transmembrane Voltage Induced by Electric Fields on Spheroidal Cells. *Biophysical Journal*, *79*(2), 670–679. https://doi.org/10.1016/S0006-3495(00)76325-9

Kotnik, T., Miklavčič, D., & Slivnik, T. (1998). Time course of transmembrane voltage induced by time-varying electric fields - A method for theoretical analysis and its application. *Bioelectrochemistry and Bioenergetics*, *45*(1), 3–16. https://doi.org/10.1016/S0302-4598(97)00093-7

Kotnik, T., Pucihar, G., & Miklavčič, D. (2010). Induced transmembrane voltage and its correlation with electroporation- mediated molecular transport. *Journal of Membrane Biology*, *236*(1), 3–13. https://doi.org/10.1007/s00232-010-9279-9

Kotnik, T., Rems, L., Tarek, M., & Miklavcic, D. (2019). Membrane Electroporation and Electropermeabilization: Mechanisms and Models. *Https://Doi.Org/10.1146/Annurev-Biophys-052118-115451*, *48*, 63–91. https://doi.org/10.1146/ANNUREV-BIOPHYS-052118-115451

Kučerka, N., Nieh, M. P., & Katsaras, J. (2011). Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidylcholines as a function of temperature. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1808*(11), 2761–2771. https://doi.org/10.1016/J.BBAMEM.2011.07.022

Leadley, C. (2008). Novel Commercial Preservation Methods. *Food Biodeterioration and Preservation*, 211–244. https://doi.org/10.1002/9780470697849.CH9

Lebovka, N. I., Praporscic, I., Ghnimi, S., & Vorobiev, E. (2005). Temperature enhanced electroporation under the pulsed electric field treatment of food tissue. *Journal of Food Engineering*, *69*(2), 177–184. https://doi.org/10.1016/J.JFOODENG.2004.08.037

Lebovka, N. I., Shynkaryk, N. V., & Vorobiev, E. (2007). Pulsed electric field enhanced drying of potato tissue. *Journal of Food Engineering*, *78*(2), 606–613. https://doi.org/10.1016/J.JFOODENG.2005.10.032

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, *55*(1–3), 181–186. https://doi.org/10.1016/S0168-1605(00)00161-6

Levkov, K., Vitkin, E., González, C. A., & Golberg, A. (2019). A Laboratory IGBT-Based High-voltage Pulsed Electric Field Generator for Effective Water Diffusivity Enhancement in Chicken Meat. *Food and Bioprocess Technology*, *12*(12), 1993–2003. https://doi.org/10.1007/S11947-019-02360-5

Li, Q., Wu, Q. Y., Jiang, W., Qian, J. Y., Zhang, L., Wu, M., Rao, S. Q., & Wu, C. Sen. (2019). Effect of pulsed electric field on structural properties and digestibility of starches with different crystalline type in solid state. *Carbohydrate Polymers*, *207*, 362–370. https://doi.org/10.1016/J.CARBPOL.2018.12.001

Li, X., Li, J., Wang, R., Rahaman, A., Zeng, X.-A., & Brennan, C. S. (2021). *Combined effects of pulsed electric field and ultrasound pretreatments on mass transfer and quality of mushrooms*. https://doi.org/10.1016/j.lwt.2021.112008

Liu, Z., Esveld, E., Vincken, J. P., & Bruins, M. E. (2019). Pulsed Electric Field as an Alternative Pre-treatment for Drying to Enhance Polyphenol Extraction from Fresh Tea Leaves. *Food and Bioprocess Technology*, *12*(1), 183–192. https://doi.org/10.1007/S11947-018-2199-X/FIGURES/6

Luengo, E., Álvarez, I., & Raso, J. (2013). Improving the pressing extraction of polyphenols of orange peel by pulsed electric fields. *Innovative Food Science & Emerging Technologies*, *17*, 79–84. https://doi.org/10.1016/J.IFSET.2012.10.005

Lyu, C., Huang, K., Yang, N., Wang, H., & Wang, J. (2016). Combination of Thermosonication and Pulsed Electric Fields Treatments for Controlling Saccharomyces cerevisiae in Chinese Rice Wine. *Food and Bioprocess Technology*, *9*(11), 1854–1864. https://doi.org/10.1007/S11947-016-1769-Z

Manzoor, M. F., Zeng, X. A., Rahaman, A., Siddeeg, A., Aadil, R. M., Ahmed, Z., Li, J., & Niu, D. (2019a). Combined impact of pulsed electric field and ultrasound on bioactive compounds and FT-IR analysis of almond extract. *Journal of Food Science and Technology*, *56*(5), 2355. https://doi.org/10.1007/S13197-019-03627-7

Manzoor, M. F., Zeng, X. A., Rahaman, A., Siddeeg, A., Aadil, R. M., Ahmed, Z., Li, J., & Niu, D. (2019b). Combined impact of pulsed electric field and ultrasound on bioactive compounds and FT-IR analysis of almond extract. *Journal of Food Science and Technology*, *56*(5), 2355. https://doi.org/10.1007/S13197-019-03627-7

Martín-Belloso, O., & Sobrino-López, A. (2011). Combination of Pulsed Electric Fields with Other Preservation Techniques. *Food and Bioprocess Technology 2011 4:6*, *4*(6), 954–968. https://doi.org/10.1007/S11947-011-0512-Z

Mason, T. J. (2016). Ultrasonic cleaning: An historical perspective. *Ultrasonics Sonochemistry*, *29*, 519–523. https://doi.org/10.1016/J.ULTSONCH.2015.05.004

Matys, A., Witrowa-Rajchert, D., Parniakov, O., & Wiktor, A. (2022). Application of pulsed electric field prior to vacuum drying: Effect on drying time and quality of apple tissue. *Research in Agricultural Engineering*, *68*(2), 93–101. https://doi.org/10.17221/93/2021-RAE

Mawson, R., Gamage, M., Terefe, N. S., & Knoerzer, K. (2011). Ultrasound in enzyme activation and inactivation. *Food Engineering Series*, 369–404. https://doi.org/10.1007/978-1-4419-7472-3_14/TABLES/2

McIntyre, M., & McNeil, B. (1997). Effects of elevated dissolved CO2 levels on batch and continuous cultures of Aspergillus niger A60: an evaluation of experimental methods. *Applied and Environmental Microbiology*, *63*(11), 4171. https://doi.org/10.1128/AEM.63.11.4171-4177.1997

Meglič, S. H., Miklavčič, D., & Vorobiev, E. (Eds.). (2021). *Pulsed Electric Fields in Biotechnology*. Frontiers Media SA. https://doi.org/10.3389/978-2-88966-936-3

Monfort, S., Gayán, E., Saldaña, G., Puértolas, E., Condón, S., Raso, J., & Álvarez, I. (2010). Inactivation of Salmonella Typhimurium and Staphylococcus aureus by pulsed electric fields in liquid whole egg. *Innovative Food Science & Emerging Technologies*, *11*(2), 306–313. https://doi.org/10.1016/J.IFSET.2009.11.007

Montanari, C., Tylewicz, U., Tabanelli, G., Berardinelli, A., Rocculi, P., Ragni, L., & Gardini, F. (2019). Heat-Assisted Pulsed Electric Field Treatment for the Inactivation of Saccharomyces cerevisiae: Effects of the Presence of Citral. *Frontiers in Microbiology*, *10*(2), 1737. https://doi.org/10.3389/FMICB.2019.01737

Munshi, M. Z. A. (1995). Handbook of Solid State Batteries and Capacitors. *Handbook of Solid State Batteries and Capacitors*. https://doi.org/10.1142/2385

Niayesh, K., & Runde, M. (2017). *Power Switching Components*. https://doi.org/10.1007/978-3-319-51460-4

Noci, F., Riener, J., Walkling-Ribeiro, M., Cronin, D. A., Morgan, D. J., & Lyng, J. G. (2008). Ultraviolet irradiation and pulsed electric fields (PEF) in a hurdle strategy for the preservation of fresh apple Juice. *Journal of Food Engineering*, *85*(1), 141–146. https://doi.org/10.1016/J.JFOODENG.2007.07.011

Nowak, D., & Jakubczyk, E. (2022). Effect of Pulsed Electric Field Pre-Treatment and the Freezing Methods on the Kinetics of the Freeze-Drying Process of Apple and Its Selected Physical Properties. *Foods*, *11*(16). https://doi.org/10.3390/FOODS11162407

Ntourtoglou, G., Drosou, F., Chatzimitakos, T., Athanasiadis, V., Bozinou, E., Dourtoglou, V. G., Elhakem, A., Sami, R., Ashour, A. A., Shafie, A., & Lalas, S. I. (2022). Combination of Pulsed Electric Field and Ultrasound in the Extraction of Polyphenols and Volatile Compounds from Grape Stems. *Applied Sciences*, *12*(12), 6219. https://doi.org/10.3390/app12126219

Ntourtoglou, G., Drosou, F., Dourtoglou, V. G., Athanasiadis, V., Chatzimitakos, T., Bozinou, E., & Lalas, S. I. (2022). Hyphenated Extraction of Valuable Compounds from Aesculus carnea: Ultrasound Extraction with Pulsed Electric Field Pretreatment. *AgriEngineering*, *4*(4), 847–854. https://doi.org/10.3390/agriengineering4040054

Ntourtoglou, G., Tsapou, E. A., Drosou, F., Bozinou, E., Lalas, S., Tataridis, P., & Dourtoglou, V. (2020). Pulsed Electric Field Extraction of α and β-Acids From Pellets of Humulus lupulus (Hop). *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.00297

Ostermeier, R., Hill, K., Dingis, A., Töpfl, S., & Jäger, H. (2021a). Influence of pulsed electric field (PEF) and ultrasound treatment on the frying behavior and quality of potato chips. *Innovative Food Science and Emerging Technologies*, *67*. https://doi.org/10.1016/J.IFSET.2020.102553

Paganin-Gioanni, A., Bellard, E., Escoffre, J. M., Rols, M. P., Teissié, J., & Golzio, M. (2011). Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(26), 10443–10447. https://doi.org/10.1073/PNAS.1103519108

Pai, S. T., & Zhang, Q. (1995). Introduction to High Power Pulse Technology. *Introduction to High Power Pulse Technology*. https://doi.org/10.1142/2311

Parniakov, O., Bals, O., Lebovka, N., & Vorobiev, E. (2016a). Pulsed electric field assisted vacuum freeze-drying of apple tissue. *Innovative Food Science & Emerging Technologies*, *35*, 52–57. https://doi.org/10.1016/J.IFSET.2016.04.002

Pataro, G., Ferrentino, G., Ricciardi, C., & Ferrari, G. (2010a). Pulsed electric fields assisted microbial inactivation of S. cerevisiae cells by high pressure carbon dioxide. *The Journal of Supercritical Fluids*, *54*(1), 120–128. https://doi.org/10.1016/J.SUPFLU.2010.04.003

Pol, I. E., Mastwijk, H. C., Bartels, P. v., & Smid, E. J. (2000). Pulsed-electric field treatment enhances the bactericidal action of nisin against Bacillus cereus. *Applied and Environmental Microbiology*, *66*(1), 428–430. https://doi.org/10.1128/AEM.66.1.428-430.2000

Pucihar, G., Kotnik, T., Miklavčič, D., & Teissié, J. (2008). Kinetics of Transmembrane Transport of Small Molecules into Electropermeabilized Cells. *Biophysical Journal*, *95*(6), 2837. https://doi.org/10.1529/BIOPHYSJ.108.135541

Puértolas, E., López, N., Condón, S., Álvarez, I., & Raso, J. (2010). Potential applications of PEF to improve red wine quality. *Trends in Food Science & Technology*, *21*(5), 247–255. https://doi.org/10.1016/J.TIFS.2010.02.002

Punthi, F., Yudhistira, B., Gavahian, M., Chang, C. K., Cheng, K. C., Hou, C. Y., & Hsieh, C. W. (2022). Pulsed electric field-assisted drying: A review of its underlying mechanisms, applications, and role in fresh produce plant-based food preservation. *Comprehensive Reviews in Food Science and Food Safety*, *21*(6), 5109–5130. https://doi.org/10.1111/1541-4337.13052

Pyatkovskyy, T. I., Shynkaryk, M. V., Mohamed, H. M., Yousef, A. E., & Sastry, S. K. (2018a). Effects of combined high pressure (HPP), pulsed electric field (PEF) and sonication treatments on inactivation of Listeria innocua. *Journal of Food Engineering*, *233*, 49–56. https://doi.org/10.1016/J.JFOODENG.2018.04.002

Raso, J., Calderón, M. L., Góngora, M., Barbosa-Cánovas, G., & Swanson, B. G. (1998). Inactivation of Mold Ascospores and Conidiospores Suspended in Fruit Juices by Pulsed Electric Fields. *LWT - Food Science and Technology*, *31*(7–8), 668–672. https://doi.org/10.1006/FSTL.1998.0426

Reineke, K., Schottroff, F., Meneses, N., & Knorr, D. (2015). Sterilization of liquid foods by pulsed electric fields-an innovative ultra-high temperature process. *Frontiers in Microbiology*, *6*(MAY), 400. https://doi.org/10.3389/FMICB.2015.00400/BIBTEX

Rhazi, N., Hannache, H., Oumam, M., Sesbou, A., Charrier, B., Pizzi, A., & Charrier-El Bouhtoury, F. (2019). Green extraction process of tannins obtained from Moroccan Acacia mollissima barks by microwave: Modeling and optimization of the process using the response surface methodology RSM. *Arabian Journal of Chemistry*, *12*(8), 2668–2684. https://doi.org/10.1016/J.ARABJC.2015.04.032

Rolong, A., Davalos, R. v., & Rubinsky, B. (2017). History of electroporation. In *Irreversible Electroporation in Clinical Practice* (pp. 13–37). Springer International Publishing. https://doi.org/10.1007/978-3-319-55113-5_2

Rubin, A. E., Levkov, K., Usta, O. B., Yarmush, M., & Golberg, A. (2019). IGBT-Based Pulsed Electric Fields Generator for Disinfection: Design and In Vitro Studies on Pseudomonas aeruginosa. *Annals of Biomedical Engineering*, *47*(5), 1314–1325. https://doi.org/10.1007/S10439-019-02225-0

Sampedro, F., Rodrigo, D., Martínez, A., Barbosa-Cánovas, G. V., & Rodrigo, M. (2016). Review: Application of Pulsed Electric Fields in Egg and Egg Derivatives. *Http://Dx.Doi.Org/10.1177/1082013206070430*, *12*(5), 397–406. https://doi.org/10.1177/1082013206070430

Sasagawa, A., Hoshino, J., Fujii, T., Yamazaki, A., Ohshima, T., Yamada, A., Kobayashi, A., & Sato, M. (2006). Inactivation of Bacillus subtilis spores by a combination of hydrostatic high-pressure and pulsed electric field treatments. *Review of High Pressure Science and Technology/Koatsuryoku No Kagaku To Gijutsu*, *16*(1), 45–53. https://doi.org/10.4131/JSHPREVIEW.16.45

Segovia, F. J., Luengo, E., Corral-Pérez, J. J., Raso, J., & Almajano, M. P. (2015). Improvements in the aqueous extraction of polyphenols from borage (Borago officinalis L.) leaves by pulsed electric fields: Pulsed electric fields (PEF) applications. *Industrial Crops and Products*, *65*, 390–396. https://doi.org/10.1016/J.INDCROP.2014.11.010

Sharma, P., Bremer, P., Oey, I., & Everett, D. W. (2014). Bacterial inactivation in whole milk using pulsed electric field processing. *International Dairy Journal*, *35*(1), 49–56. https://doi.org/10.1016/J.IDAIRYJ.2013.10.005

Sharma, P., Oey, I., Bremer, P., & Everett, D. W. (2018). Microbiological and enzymatic activity of bovine whole milk treated by pulsed electric fields. *International Journal of Dairy Technology*, *71*(1), 10–19. https://doi.org/10.1111/1471-0307.12379

Shayanfar, S., Chauhan, O., Toepfl, S., & Heinz, V. (2014). Pulsed electric field treatment prior to freezing carrot discs significantly maintains their initial quality

parameters after thawing. *International Journal of Food Science and Technology*, *49*(4), 1224–1230. https://doi.org/10.1111/IJFS.12421

Silve, A., Kian, C. B., Papachristou, I., Kubisch, C., Nazarova, N., Wüstner, R., Leber, K., Strässner, R., & Frey, W. (2018). Incubation time after pulsed electric field treatment of microalgae enhances the efficiency of extraction processes and enables the reduction of specific treatment energy. *Bioresource Technology*, *269*, 179–187. https://doi.org/10.1016/J.BIORTECH.2018.08.060

Simon, P., & Gogotsi, Y. (2008). Materials for electrochemical capacitors. *Nature Materials*, *7*(11), 845–854. https://doi.org/10.1038/NMAT2297

Sitzmann, W., Vorobiev, E., & Lebovka, N. (2017). Pulsed electric fields for food industry: Historical overview. In *Handbook of Electroporation* (Vol. 4, pp. 2335–2354). Springer International Publishing. https://doi.org/10.1007/978-3-319-32886-7_194

Spilimbergo, S., Dehghani, F., Bertucco, A., & Foster, N. R. (2003a). Inactivation of bacteria and spores by pulse electric field and high pressure CO2 at low temperature. *Biotechnology and Bioengineering*, *82*(1), 118–125. https://doi.org/10.1002/BIT.10554

Spilimbergo, S., Dehghani, F., Bertucco, A., & Foster, N. R. (2003b). Inactivation of bacteria and spores by pulse electric field and high pressure CO2 at low temperature. *Biotechnology and Bioengineering*, *82*(1), 118–125. https://doi.org/10.1002/BIT.10554

Stefan Toepfl and Dietrich Knorr. (2008). Pulsed electric fields as a pretreatment technique in drying processes. *Stewart Postharvest Review*, *2*(4), 1–6. https://doi.org/10.2212/SPR.2006.4.3

Telfser, A., & Gómez Galindo, F. (2019). Effect of reversible permeabilization in combination with different drying methods on the structure and sensorial quality of dried basil (Ocimum basilicum L.) leaves. *LWT*, *99*, 148–155. https://doi.org/10.1016/J.LWT.2018.09.062

Tomasula, P. M., Craig, J. C., & Boswell, R. T. (1997). A continuous process for casein production using high-pressure carbon dioxide. *Journal of Food Engineering*, *33*(3–4), 405–419. https://doi.org/10.1016/S0260-8774(97)00053-8

Tylewicz, U. (2020). How does pulsed electric field work? *Pulsed Electric Fields to Obtain Healthier and Sustainable Food for Tomorrow*, 3–21. https://doi.org/10.1016/B978-0-12-816402-0.00001-X

Vorobiev, E., & Lebovka, N. (2020). Processing of foods and biomass feedstocks by pulsed electric energy. In *Processing of Foods and Biomass Feedstocks by Pulsed Electric Energy*. Springer International Publishing. https://doi.org/10.1007/978-3-030-40917-3

Walkling-Ribeiro, M., Noci, F., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2009). Shelf life and sensory evaluation of orange juice after exposure to thermosonication and pulsed electric fields. *Food and Bioproducts Processing*, *87*(2), 102–107. https://doi.org/10.1016/J.FBP.2008.08.001

Walkling-Ribeiro, M., Noci, F., Riener, J., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2009). The impact of thermosonication and pulsed electric fields on Staphylococcus aureus inactivation and selected quality parameters in orange juice. *Food and Bioprocess Technology*, *2*(4), 422–430. https://doi.org/10.1007/S11947-007-0045-7/METRICS

Wei, Y., Xiao, Y., & Wang, Z. (2021). The interventional therapeutics of irreversible electroporation for pancreatic cancer. *Integrative Pancreatic Intervention Therapy*, 355–362. https://doi.org/10.1016/B978-0-12-819402-7.00014-0

Wiktor, A., Parniakov, O., & Witrowa-Rajchert, D. (2022). Drying Improving by Pulsed Electric Fields. *Food Engineering Series*, 385–397. https://doi.org/10.1007/978-3-030-70586-2_13/COVER

Williamson, J., Shanahan, M., & Hochmuth, R. (1975). The Influence of Temperature on Red Cell Deformability. *Blood*, *46*(4), 611–624. https://doi.org/10.1182/BLOOD.V46.4.611.611

Wu, Y., & Zhang, D. (2014). Effect of pulsed electric field on freeze-drying of potato tissue. *International Journal of Food Engineering*, *10*(4), 857–862. https://doi.org/10.1515/IJFE-2014-0149/MACHINEREADABLECITATION/RIS

Xia, Q., Liu, Q., Denoya, G. I., Yang, C., Barba, F. J., Yu, H., & Chen, X. (2022). High Hydrostatic Pressure-Based Combination Strategies for Microbial Inactivation of Food Products: The Cases of Emerging Combination Patterns. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/FNUT.2022.878904

Yarmush, M. L., Golberg, A., Serša, G., Kotnik, T., & Miklavčič, D. (2014). Electroporation-based technologies for medicine: Principles, applications, and challenges. *Annual Review of Biomedical Engineering*, *16*, 295–320. https://doi.org/10.1146/ANNUREV-BIOENG-071813-104622

Yogesh, K. (2016). Pulsed electric field processing of egg products: a review. *Journal of Food Science and Technology*, *53*(2), 934. https://doi.org/10.1007/S13197-015-2061-3

Yu, Y., Jin, T. Z., Fan, X., & Xu, Y. (2017). Osmotic dehydration of blueberries pretreated with pulsed electric fields: Effects on dehydration kinetics, and microbiological and nutritional qualities. *Https://Doi.Org/10.1080/07373937.2016.1260583*, *35*(13), 1543–1551. https://doi.org/10.1080/07373937.2016.1260583

Yu, Y., Jin, T. Z., & Xiao, G. (2017). Effects of pulsed electric fields pretreatment and drying method on drying characteristics and nutritive quality of blueberries. *Journal of Food Processing and Preservation*, *41*(6), e13303. https://doi.org/10.1111/JFPP.13303

Zeng, F., Gao, Q. Y., Han, Z., Zeng, X. A., & Yu, S. J. (2016). Structural properties and digestibility of pulsed electric field treated waxy rice starch. *Food Chemistry*, *194*, 1313–1319. https://doi.org/10.1016/J.FOODCHEM.2015.08.104

Zhang, Q., Barbosa-Cánovas, G. v., & Swanson, B. G. (1995). Engineering aspects of pulsed electric field pasteurization. *Journal of Food Engineering*, *25*(2), 261–281. https://doi.org/10.1016/0260-8774(94)00030-D

Zimmermann, U. (1982). ELECTRIC FIELD-MEDIATED FUSION AND RELATED ELECTRICAL PHENOMENA. In *Biochimica et Biophysica Acta* (Vol. 694).

Zimmermann, U. (1986). Electrical breakdown, electropermeabilization and electrofusion. *Reviews of Physiology, Biochemistry and Pharmacology*, *105*, 176–256. https://doi.org/10.1007/BFB0034499/COVER

Δημοσίευση Ι

Pulsed Electric Field Extraction of α and β-Acids from Pellets of *Humulus lupulus* (Hop)

George Ntourtoglou[1], Evangelia Anastasia Tsapou[1], Fotini Drosou[1], Eleni Bozinou[2], Stavros Lalas[2], Panagiotis Tataridis[1] and Vassilis Dourtoglou[1]*

1 Department of Wine, Vine and Beverage Sciences, University of West Attica, Athens, Greece,
2 Department of FoodScience and Nutrition, University of Thessaly, Karditsa, Greece

ABSTRACT

This paper investigates the process of extracting hop pellets (hops) utilizing the pulsed electric field (PEF) technique and the contrasting effects of the technique between two distinct hop varieties (one bitter and one aromatic). The effect of PEF on the extraction was evaluated by measuring the concentration ofα-acids andβ-acids (humulones and lupulones). Regarding the aromatic character, the hop's volatile caryophyllene, humulene and β-myrcene were analyzed both with and without employing the PEF treatment. In order to analyze the acids and the volatile fraction, the analytical method of UV-Vis spectrophotometry was applied followed by gas chromatography coupled with mass spectrometry. For the second technique, the extracts were previously purified through a Graphitized Carbon Black syringe for Solid Phase Extraction. The results revealed that PEF had a positive impact on the alpha acids of bitter hops by increasing the extraction rate of these acids by 20%, while the volatiles demonstrated an increase of 5.6 and 7.4% for humulene and caryophyllene, respectively. Concerning the aromatic variety of hops, the PEF treatment appeared to have no noteworthy effects.

Keywords: hops, pulsed electric field, α-acids, β-acids, extraction, SPE

INTRODUCTION

Hop pellets are renowned for contributing to the bitterness of the taste and the enrichment of aroma in beer. They come from the plant *Humulus lupulus* and, specifically, from its female cone. The genus is represented by two species; the Humulus, the common hops (*H. lupulus L.*), and the Japanese hops (*H. japonicus Sieb.* and *Zuce.*). The Humulus genus particularly, belongs to the family of the Cannabinaceae (Steinhaus and Schieberle, 2000). Hops complement beer in a complex way due to their chemical composition, which varies depending on the variety used, the

cultivation techniques and the extraction that occurs during the processing of the beer. In air-dried hop cones, water accounts for 10%, total resins for 15%, essential oils 0.5–2%, tannins 4%, monosaccharides 2%, proteins 15%, ash 8% and cellulose 43% (Stevens, 1967). Additionally, it is remarkable to mention that hops have been used in folk medicine in the past since they possessa broad spectrum of medico-pharmaceutical properties. The hop pellets are financially exploited primarily by the beer industry. Hop resins include hard resins, soft resins and uncharacterized resins. Hard resins make up the part of the total resins which is insoluble in low boiling paraffinic solvents. Soft resins contrastively, are the fraction of total resins soluble in low boiling paraffinic hydrocarbons and mainly include the acids and theβ-acids. Theα-acids consist of humulones, cohumulones and adhumulones while the β-acids include lupulones, colupulones and adlupulones (Stevens, 1967). There is also another part of the resins which are uncharacterized. This fraction is the portion of the soft resin remaining after precipitation of α-acids with lead acetate and crystallization of β-acids (Stevens, 1967; Kunze, 2004). Another important constituent of the hop flower, is the essential oils located within the hop cone. This fraction is also known as “hop oil” and is mainly composed by the volatile aromatic compounds. The total oil content depends on thevariety of hop and varies between 0.1 and 2.0% by dry weight (Stevens, 1967). In this fraction more than 400 hop flavor components have been identified in majority monoterpenes (C10) and sesquiterpenes (C15). The main volatiles in hops cultivars are myrcene, α-humulene andβ-caryophyllene, which account for80% (Rettberg et al., 2018). Myrcene varies from variety to variety and can contain from 10 to 72% of the “hop oil”. This compound bestows the green fresh note with resinous aspects (Steinhaus and Schieberle, 2000; Nance et al., 2011). Myrcene’s oxidation forms many terpenoids, such as linalool, geraniol, citral, a-terpineol and carvone, known for their augmenting effects on the aroma (Dieckmann and Palamand, 1974; Rettberg et al., 2018). With respect toα-humulene (15–42% of the essential oil of hops) andβ-caryophyllene (2.8–18.2% of the essential oil), they are known for their woody and spicy odor (Peacock and Deinzer, 1981;Nickerson and Van Engel, 1992; Peacock and McCarty, 1992;Eyres and Dufour, 2009).Conventional extraction methods require extended extraction times, high purity solvents, often offer low extraction selectivity and, finally, in some cases are responsible for the thermal decomposition of sensitive compounds (Bozinou et al., 2019).For the above reasons, new extraction techniques have been introduced. These methods include ultrasonic waves (Hossainet al., 2014; Bimakr et al., 2017), gamma irradiation (Gyawaliet al., 2006; Pinela et al., 2014) and electric fields, including the pulsed electric field (PEF) (Delsart et al., 2012; Zhang et al., 2012).These methods are already applied to other crops of commercial interest such as grapes, onions, potatoes, etc. New technologies, such as PEFs and high voltage electric discharges (HVED), have been proposed formicrobialin activation of food liquids (Delsart et al., 2015), for the extraction of compounds from Chardonnay grapes (Grimi

et al.,2009) or other fruits, such as apples (Grimi et al., 2011). HVEDshave also been proposed for the extraction of polyphenols and other compounds with antioxidant activity (Boussetta et al.,2009; Boussetta et al., 2011).The application of PEF has principally been used as a non-thermal treatment of liquid foods aiming to the inactivation of microorganisms (Grahl and Markl, 1996; Alvarez et al., 2003).The microbial inactivation is a function of food composition which depends on the composition of the solution and the electrical parameters (Heinz et al., 2003; Touya, 2005; Vorobievand Lebovka, 2010). Other researchers have introduced electric field treatment for the acceleration of aging of young wine thanks to the extraction of flavor compounds from wood (Zeng et al.,2008; Drosou et al., 2017).The disruption of the cell membrane due to electroporation is caused by the high intensity of the fields induced by PEF. This disturbance of the architectural structure of the membrane and the disorganization of the integrity of microbial or plant cells, lead to complex phenomena such as cell lysis or the fusion of protoplasts. When the transmembrane potential exceeds acritical value, generally around 0.8 to 1 V (Zimmermann,1986), pore formation occurs in the cell membrane and certain metabolites diffuse in the extracellular medium. This state can be transient and reversible if the applied field remains below a certain level (Cukjati et al., 2007). On the other hand, the electropermeabilization of cells must be irreversible when the objective is the inactivation of microbial cells. The PEF treatment system is not a simple device and consists of a high voltage source, in some cases a capacitor bank, a switch and the treatment chamber. The PEF treatment chamber comprises two or more electrodes, filled with the material to be treated and it is constructed so that the electric field acting on the mass of the product to be treated is as homogeneous as possible (Maged and Ayman, 2012). Hops are the most complex and costly raw material used in brewing. Of all the herbs that have been used to flavor and preserve beer over the ages, only the hop (*H. lupulus L.*) is now regarded as an essential raw material in brewing throughout the world. In 2017, 148,603 tons of hops were produced worldwide(FAO). The majority was produced in the United States, with a total of 47,000 tons. Considerable amounts were also produced in Ethiopia and Germany yielding up to 38,000 and32,000 tons, respectively. The estimated needs for α-acids are calculated up to 8,000 tons, and the average price is valued approximately at 8 United States $ per kg. Demands for alpha acids are estimated on the basis that an average of 4.1 g is needed per hectoliter of beer (European Commission). Hop content varies depending on the type of beer, particularly considering its bitterness, and the variety of hop used. Hop content displays a steady decline in percentage annually (it still stood at 6.3 g alpha per hectoliter in 1995) due to the consumers' growing preference for less bitter beers, and the technological progress that this preference has brought about. Different brewing techniques have been developed to enhance the extraction of volatiles and acids in hopped beers. The most significant contribution of hops to beer flavoring is that of the so-called soft resins, principally the alpha acids (also known as

humulones), which are ultimately responsible for the characteristic bitterness in the taste. Analytically, the aroma of hops and the flavor of hoppy beers cannot be measured by the quantification of a single odorant; moreover, the selection of several key compounds or a comprehensive characterization (profiling) is of great importance. Analysis of hops and beer is challenging. This study determines the effect of PEF treatment on two hop varieties for the extraction of bitter acids and volatiles. No previous studies have been published on this field to the extent of our knowledge. Additionally, research on the divergent effects of the treatment on two separate hop varieties (bitter and aromatic), was carried out.

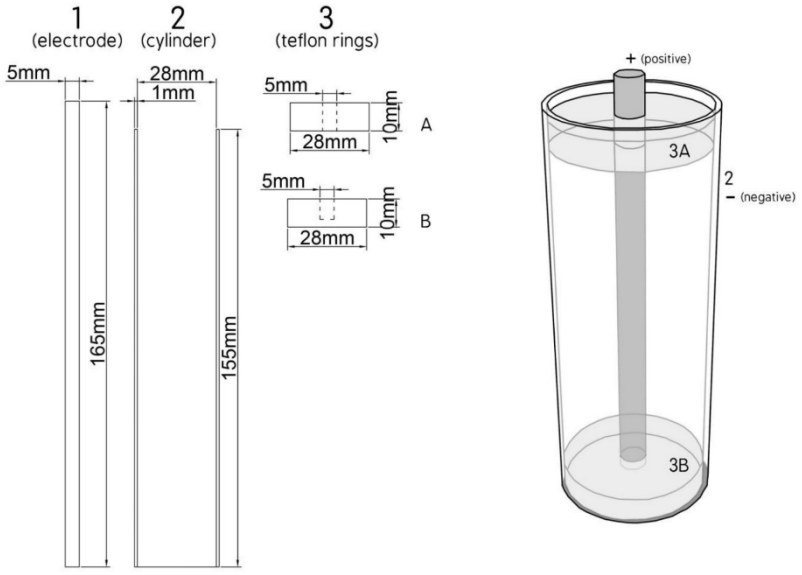


FIGURE 1 | View of treatment chamber.

MATERIALS AND METHODS

Plant Material

The two different varieties of hop cones (pelletized) used in this study were purchased by the Macedonian Thrace Brewery S.A.(Athens, Greece). The first variety was bitter, known for its high content in “bitter” acids. The second variety was aromatic, known for its high quality of essential oils. The characteristics of the two varieties were determined (using the methods described below).

Moisture Content Determination

For the determination of the dry matter content of hops, an established method regarding the moisture content of hops and hop products by European Brewery Convention, 2006 (EBC,7.2, 1998) was employed. After being weighted, the samples

were dried in a vacuum oven in 85◦C for 6 h. The moisture percentage was determined according to the following equation: Moisture in, hops was calculated as: %=$\frac{loss\ in\ wt \times 100}{\text{wt of sample}}$(loss inwt×100)/(wt of sample).

Hop Storage Index (HSI)

The determination of HSI was carried out according to the American Society of Brewing Chemists and specifically the Method of Analysis HOPS-6. A, where, the oxidative decrease in bothα- andβ-acids content during storage is determined by the progressive increase in the ratio of absorbance at 275–325 nm. Such loss inα- andβ-acids and increase in the hopstorage index (HSI) ratios may reflect unfavorably on the utility and quality of the hops.

The HSI was calculated on a ratio of absorbance at 275 nm(A275) to the absorbance at 325 nm (A325) after PEF treatment and compared to the same ratio without PEF (control).

Chemicals The dichloromethane, chloroform, sodium chloride, ethylacetate, methanol, N-pentane, anhydrous sodium sulfateand 2-octanol used were purchased from the Chem Lab(Zedelgem, Belgium).

PEF Equipment

The PEF equipment used was provided by Val-Electronic (Athens, Greece), and included the static bench scale system, reported previously (Bozinou et al., 2019), accompanied by another high voltage power generator (from Eisco, India). The model of the batch processing chamber (TC) was adapted froma design of cylinder type electrodes (Ohshima and Sato, 2004) and consists of a coaxial stainless steel electrode [5 mm in diameter and 165 mm high, Figure 1(1)] placed inside a bronze cylinder [1 mm thick, 155 mm high and 30 mm outer diameter Figure 1(2)] with a closed flat bottom. In this cylinder are placed two teflon rings (28 mm diameter and 10 mm thick Figure 1(3), one at the bottom Figure 1(3B) and another at the top Figure 1(3A) with a hole in the middle to pass the electrode which serve to isolate the electrode of the outer bronze cylinder (Figure 1).The electric field strength E is evaluated as E= U/d, where “U” is the applied voltage and “d” is the distance between the electrode and the bronze cylinder (d= 12.4 mm).For each case, the treatment was calculatedas:

$$t = (ti + tp) \times P$$

ti = pulse duration (sec)

tp = pause time (sec)

P= number of pulses.

For GC-MS analysis the extraction solvent was methanol. For UV-Vis analysis the extraction solvents were methanol, toluene and water. For the capacitance measurement the solvents were methanol, water and ethanol. For the extraction method with water the electric field strength was E = 2.42 kV/cm, t = 30 min (ti = 1 μs, tp = 1 s, 1800 pulses), while for all the others solvents the treatment conditions were E = 1.13 kV/cm, t = 30 min (ti = 1 μs, tp = 1 s, 1800 pulses). For the combination “treatment chamber- sample” there was no dielectric breakdown until the electric field strength of 2.5 kV/cm for 56 μF capacitance of the discharge capacitor. On these grounds, 1.15 to 2.5 kV/cm were used during this work. In order to select the number of pulses, UV-Vis determinations were utilized to quantify the difference between treated samples and controls. Absorbance was measured every 900 pulses. Accordingly, the number of pulses selected was 1800. The pulse width was approximately 1 μs and the frequency of the pulse was 1 Hz. Treatment time was 0.75 ms. The temperature raise caused by the treatment was negligible (<1◦C).

Sample Preparation for PEF and ControlTreatments

Around 6 g of hop pellets were grounded to a fine powder using a grinding bowl. A 2.5 g amount of this powder was weighed in a Schott Duran laboratory bottle (100 mL) with Teflon-lined screw cap, and then, 50 mL of methanol was added (same procedure for the other solvents). For the control, another identical sample was prepared and both they left at 25◦C for 30 min. Then, one sample was transferred to the treatment cell for PEF and the other was used for control. After the treatment (30 min) both samples were gravity filtered to remove the plant material and the filtrate (hop extract) was transferred into a vial (20 mL) for analysis as described below.For the experiment with hydrated hop pellets, an additional step was added for the two samples (treated and control) which consist of a 30-min hydration in HPLC water before treatment in methanol or water.For the evaluation of the treatment time on the extractability of the acids, the extraction medium was methanol. Treatments of 15, 30, 45, and 60 min (increments of 900 pulses) were performed. And at the end of each time, the treated hop pellets were filtered and processed as described above. The same procedure was also carried out for the control sample.In all treatments, care must be taken to keep the temperature below the boiling point of the solvent used.

α- and β-Acids Determination Using UV-Vis Spectra

The method used was adapted from Alderton et al. (1954) and Egts et al. (2012). Specifically, in a 25 mL volumetric flask, 50 μL of the filtrate was added to a methanolic solution of NaOH (0.5 mL of 6M NaOH in 250 mL of methanol) and the

complete spectrum (520 to 210 nm) was recorded against a solution of methanol in methanolic NaOH (50 μL:25 mL) as a blanc. The formulas used to find α-acid, β-acid and a third component (comp 3) are the following:

$$A355 = 31.8C\alpha + 46.0C\beta + 1.0Ccomp3$$

$$A325 = 38.1C\alpha + 33.1C\beta + 1.5Ccomp3$$

$$A275 = 9.0C\alpha + 3.7C\beta + 3.1Ccomp3$$

where A355, A325, and A275 stand for the absorbance of the three analytical wavelengths and Cα, Cβ, and Ccomp3 stand for the concentrations (in mg/L) of the α-acids, β-acids, and the third component, respectively (Egts et al., 2012).

α-Acids, β-Acids and Terpenes,Determination Using GC-MS

Prior to GC-MS analysis, the hop extracts were purified by applying a solid phase extraction treatment (SPE) using a graphite carbon black syringe (GCB). The syringe was first washed with 10 mL of dichloromethane (DCM) and then conditioned with 10 mL of methanol and 10 mL of deionized water under vacuum to the point of complete dryness. After that, 5 mL of methanolic hop extract was added with 3 mL of distilled water to the GCB syringe. The vacuum was then adjusted to give a flow of 10 mL/min and the cartridges were dried under full vacuum for 10 min. When the cartridges were dried, they were eluted with 5 mL ethyl acetate and 5 mL DCM. The eluents were collected, then dried over sodium sulfate and filtered before adding 50 μL of the internal standard (2-octanol 2500 ppm diluted in the pentane). The sample was then concentrated into a flash evaporator to 1 mL and 1 μL of the sample was injected to the GC-MS.

Capacitance of the Treatment Chamber

In order to measure the capacitance of the treatment chamber, the chamber was consecutively filled with the materials used in the experiments. To achieve a correct capacitance measurement, the treatment chamber must be electrically discharged. For each of the materials the value of the capacitance was measured with a digital capacitance meter (ProsKit MT-5110, Prokit's IndustriesCo. Ltd., Taiwan) with precision ± 0.5%.

Gas Chromatography/MassSpectrometry Analysis

The instrumentation, the column and the conditions of GC-MS used were previously described by Drosou et al. (2017).

DPPH• Assay

The antioxidant activity of hop extracts was determined using the DPPH• assay. A slightly modified method of Blois (1958) was adopted. At first, the samples were properly diluted in methanol or ethanol (1:10). An aliquot of 0.1 mL of each diluted extract was added to 3.9 mL of DPPH• radical solution (0.0029 g/100 mL methanol) and the solution was then vortexed. After 20 min of remaining in the darkness, the absorbance of each mixture was measured at 515 nm. Pure methanol with the DPPH• radical was used as control. All samples were prepared in triplicate. Percentage of inhibition of DPPH• radical I (%) of each hop extract was calculated according to the following equation:

$$I(\%) = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} * 100$$

where Ablank stands for the absorbance of DPPH• with methanol instead of sample and

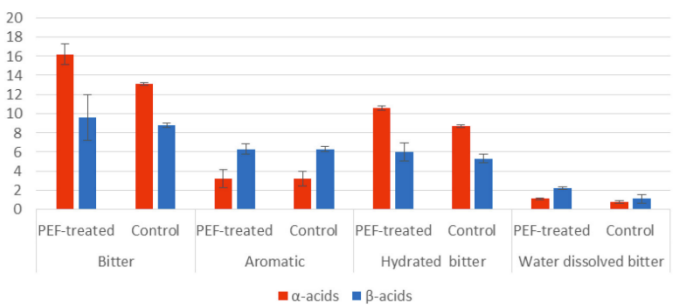


FIGURE 2 | % extraction of α-acids and β-acids determined by UV–vis spectrophotometer.

Asample is the absorbance of DPPH• after the reaction with hop extracts.

Statistical Analysis

Results are displayed as means of triplicate determinations. Statistical analysis was

TABLE 1 | Hop moisture, hop storage index (HSI), α- and β-acids content and α-acids losses.

	Hop variety	
	Aromatic	**Bitter**
Moisture (%)	9.47	9.06
HSI	0.43	0.29
α-acids (%)	2.30	10.80
β-acids (%)	3.20	8.40
α-acids losses (%)	105.36	91.87

carried out using the Excel 2013 (Microsoft, United States) software. Standard deviation for the concentrations of α- and β-acids was calculated and presented in **Tables 5, 6** and in **Figure 2.**

Risk Assessment

Treatments in organic solvents should be done with caution. In general, in the absence of water or even in binary water- flammable organic solvent systems, the flash point of the solvent or mixture should be taken into account; the temperature should not be increased, preferably at room temperature and the treatment should be carried out in closed systems. Avoid electrical sparks around the treatment chamber.

RESULTS AND DISCUSSION

Hop Storage Index (HSI)

The physical and chemical values of the two varieties were determined prior to the PEF treatment in order to acquire knowledge on the composition of hop samples. As it was reported by Roberts (2016), HIS is a measure of the degradation and can be used to quantify the losses of α-acids and β-acids during treatment. As it is shown in **Table 1**, HIS values (0.3–0.4) in bitter and aromatic hop are low, indicating a fresh raw material, as stated by Van Holle et al. (2017). Following PEF treatment, extracts were subjected to an analysis based on their UV-Vis spectra.

TABLE 2 | Analysis of spectra from UV–vis.

		Bitter, methanol extracted		Aromatic, methanol extracted		Bitter, hydrated and methanol extracted		Bitter, hydrated and water extracted	
		PEF-treated	Control	PEF-treated	Control	PEF-treated	Control	PEF-treated	Control
Absorbance in nm	275	0.534	0.396	0.246	0.242	0.345	0.266	0.161	0.104
	325	0.918	1.153	0.425	0.422	0.712	0.589	0.185	0.102
	355	0.903	1.139	0.455	0.452	0.686	0.575	0.183	0.097
Acids	α-acids	16.2%	13.1%	3.2%	3.2%	10.6%	8.7%	1.1%	0.8%
	β-acids	9.6%	8.8%	6.3%	6.3%	6%	5.3%	2.2%	1.1%
Increase with PEF treated for	α-acids	24%		0%		21%		100%	
	β-acids	9%		1%		14%		120%	
HSI		0.58	0.34	0.57	0.57	0.48	0.44	0.87	1.01

As it can be observed (**Table 1**) the bitter hop variety had a 249% higher concentration of total acids compared to the aromatic one. The α-acids are the precursors of iso- α-acids which are formed in the boiling wort resulting in the bitterness of beer. Specifically, the α-acids had 370% higher concentration compared to the aromatic, while for β-acids the concentration was 162%. The results above, partially clarify the difference observed in the extractability using PEF. After PEF treatment, the HSI level varied with the extraction media. In samples treated with methanol the value was 0.58, while for those treated with water, it was 0.87 (0.34 and 1.01 for the control sample, respectively) (**Table 2**). It appears quite evidently, that following the processes of extraction and treatment with PEF, there is an increase in HSI in both bitter and aromatic varieties but in insignificantly low values indicating that PEF has not any deleterious effects in the raw material. These values of course, are merely results of comparison between hop samples and not between different extraction media, in which differences of solubility drastically influence the final result.

Effect of PEF on the Extractability of α- and β-Acids

Hop pellets from the *H. lupulus* plant contain both α-acids (humulones) and β-acids (lupulones) as well as many other compounds that interfere in the UV-Vis spectrum (**Figures 2, 3**). The isomerization of α-acids to iso-α-acids during boiling is a process which strongly influences the taste of beer. The iso- α-acids are responsible for the distinct bitterness of the taste. The positive effect of the PEF treatment lies in the increase in the extractability of the α-acids which are then isomerized into iso- α-acids. The method applied for visualizing the PEF effect is a three-component analysis (Egts et al., 2012; **Figure 3**). Therefore, in order to determine the impact of the samples of hops treated with PEF, a spectrophotometric plot was followed to quantify the acids α and β, as well as those of the third component (iso-α-acids, etc.).

The spectra obtained from UV-Vis plot are shown in **Figures 3A,B** and the calculated results are presented in **Table 2**. The spectra presented refer to treatment of bitter hop

in methanol and in water. The decrease of the absorbance is attributed to the acids' low solubility in water; nevertheless, the shape of the curve is the same in both treatment media, where the hop exhibits a similar physical and chemical response.

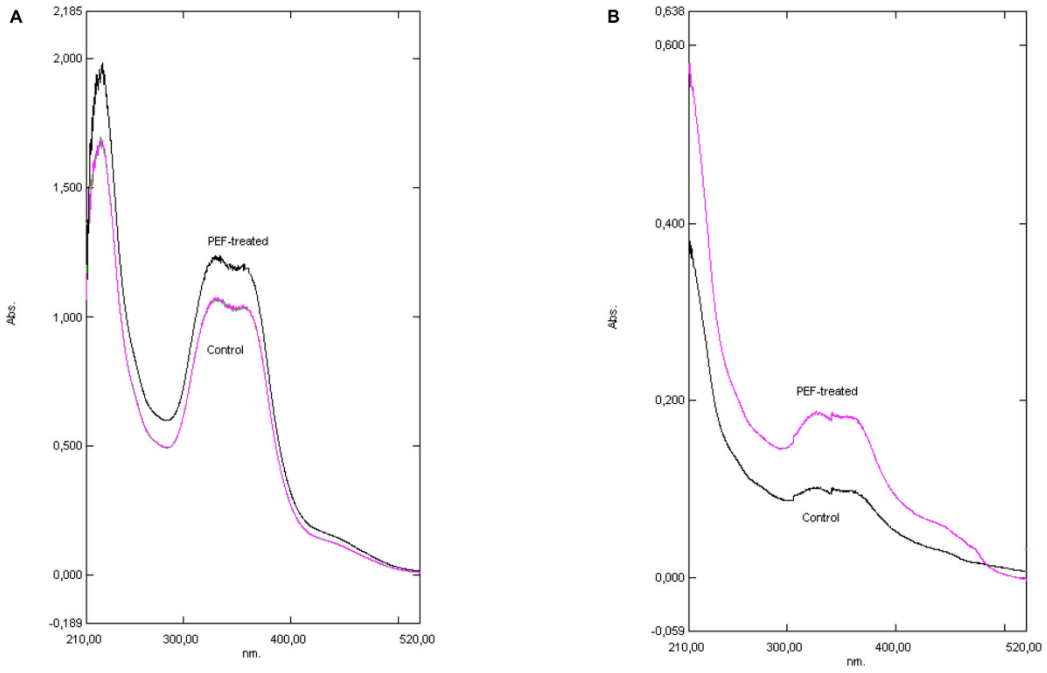


FIGURE 3 | The effect of extraction media. Basic methanolic **(A)** versus Water **(B)**. For the quantification of the acids' absorbance at 275, 325, and 355 nm was used. **(A)** The UV–vis spectrum of both samples, PEF treated and Control (bitter hop was suspended in methanol). **(B)** The UV–vis spectrum of both samples, PEF treated and Control (bitter hop was suspended in water).

The spectra obtained from the UV-Vis plot are presented in **Figures 3A,B** and the calculated results are presented in **Table 2**. The spectra presented refer to the treatment of bitter hop pellets in methanol and in water. The decrease in absorbance is attributed to the low solubility of acids in water; however, the shape of the curve is the same in both processing media, where the hop pellets have a similar physical and chemical response.

TABLE 3 | Content (mg/L) in α-acids and β-acids of toluene extracted bitter hop with UV–vis analysis.

Acids	Control	PEF treated	Difference
α-acids	11.8	25.8	118.03%
β-acids	7.8	14.5	85.39%

According to the results shown in **Table 2**, the difference of theα-acids and β-acids regarding the bitter hop are ranked between 9.1 and 23.7%. More specifically, the α-acids of the PEF treated sample were 23.7% higher than those of the control displaying in this manner the positive aspects of this treatment. As it has already been mentioned, humulones are isomerized into iso- α-acids, while the β-acids (also 9.1% higher) are mostly oxidized rather than isomerized. It was also observed, by employing different extraction media (solvents) or different varieties of hop, the results exhibited significant deviations **(Figure 2).**

TABLE 4 | Capacity of PEF treatment chamber with different solvents.

Sample	Capacitance (μF)	Water (mL)/methanol or ethanol (mL)/plant material (gr)
Water suspended hop	56.0	50/0/2.5
Methanol with hydrated hop	27.9	25/25/2.5
Methanol with dried hop	14.9	0/50/2.5
Ethanol with hydrated hop	24.2	25/25/2.5
Ethanol with dried hop	54.0	0/50/2.5

The aromatic hop (low concentration of α-acids and β-acids), under the same experimental conditions, showed no differences in PEF treatment and control. Finally, in order to examine the significance of the absence of water in the dried hop, the hops were hydrated for 30 min before treatment. This process produced similar results but with lower concentration in acids in comparison with the sample that was not hydrated. The aforementioned results are summarized in **Tables 2, 3**. During this process, methanol was used as solvent with the purpose to evaluate the PEF treatment. The extractability in methanol is intermediate between non-polar solvents like toluene and polar solvents like water.

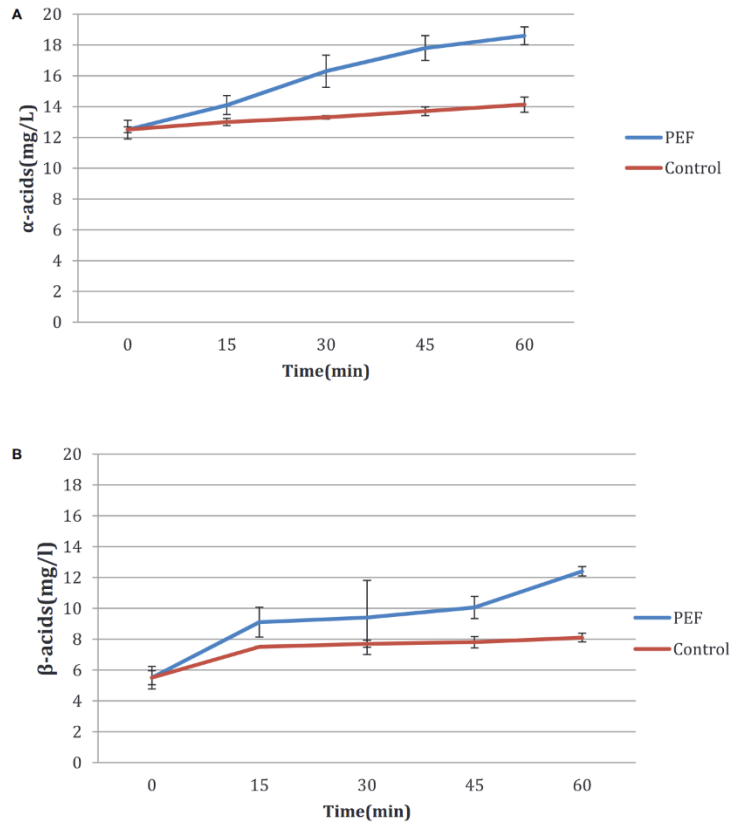


FIGURE 4 | (A,B) Influence of time of treatment to bitter hop samples.

Capacity of PEF Treatment Chamber and the Effect of Extraction Media

When the processing chamber is filled with a liquid or solid element, it becomes a capacitor. The electrodes become the conductors and the sample, which is being processed, the dielectric. The higher the conductivity of the product, the easier the electrical current flows. Thus, for high conductivity samples, a lower voltage should be used to avoid sparks. The capacity in water is much higher than the electric capacity in solvents such as methanol or ethanol **(Table 4)**. Most studies in the literature that assess the composition and release of hop ingredients in wort have been carried out with solvents such as methanol or other non-polar solvents (pentane and toluene). During the production of beer, the extraction of the hop constituents takes place in an almost hydro environment. In view of this, this study was carried out using aqueous media combined with pure methanol. The hop pellets were hydrated with pure HPLC water, and then suspended in methanol or pure water before treatment with PEF. In all these environments, the capacity of the processing cell was measured. The hydrated hop results **(Figure 3B)** showed a week absorbance after PEF treatments across all spectra. By comparing the percentage of α- and β-acids extracted from

hydrated bitter hops, we can conclude that due to their insolubility, the concentration of humulones and lupulones was much lower for PEF and the control samples and, consequently, their absorbance showed lower values. However, by examining their differences in percentages **(Table 2)**, it can be concluded that the relative extractability due to PEF in water of acids and other compounds is higher than in non-polar solvents.

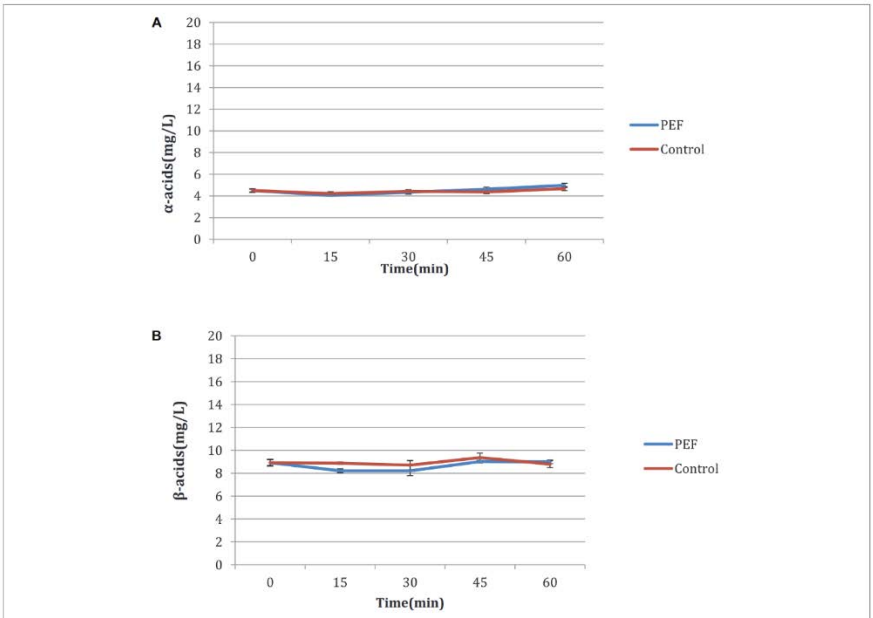


FIGURE 5 | (A,B) Influence of time of treatment to aromatic hop samples.

Time Influence

An additional experiment was carried out to measure the influence of the duration of the PEF, as well as the differences between the two varieties, aromatic and bitter. It is

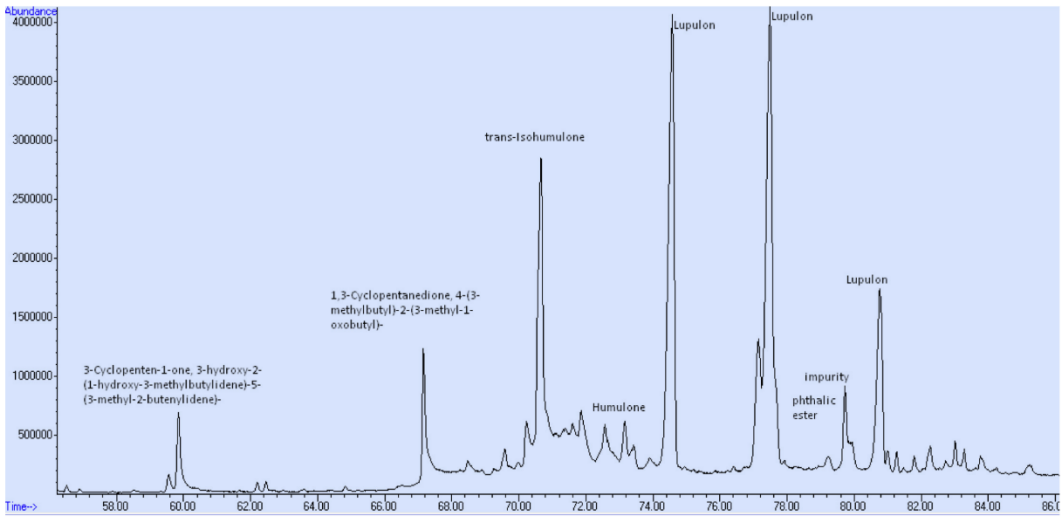


FIGURE 6 | GC-MS analysis after purification thought SPE.

observed that in the bitter, the acid concentration increases with the treatment time **(Figures 4A,B)** with or without PEF treatment. The variety of hops seems to have a significant effect on the final results. As shown in **Figures 5A,B**, the aromatic hops treated with PEF did not show any particular difference compared to the control. In addition, over time, the control samples and the samples treated with PEF have a negligible increase in the concentration of α and β-acids. We can deduce that in aromatic hops, the acids are mainly in free form unlike bitter, in which an amount of acids is probably localized in plant cells and is released after cell rupture with PEF.

Volatile Analysis

The impact of the application of PEF in volatile compounds of bitter hops is presented in Table 5, while the average of the α and β-acids from the GC-MS analysis (compared to the spectrophotometric analysis) in **Table 6**. In order to avoid contamination of the GC column, an intermediate purification step was applied, using a solid phase extraction column (SPE). This purification step was carried out in order to avoid liquid-liquid extraction of the treated samples and thus avoid deterioration of the GC columns with waxes and non-volatile hop resins (Stevens, 1967; American Society of Brewing Chemists, 1992, Eri et al., 2000). The hops are rich in resins which are easily extracted by non-polar solvents and thus deteriorate the GC columns during analysis.

Myrcene, β-caryophyllene and α-humulene are the most abundant terpenes in hops. In dry granules, their aroma is persistent and characteristic. In beer, they are lost as odor

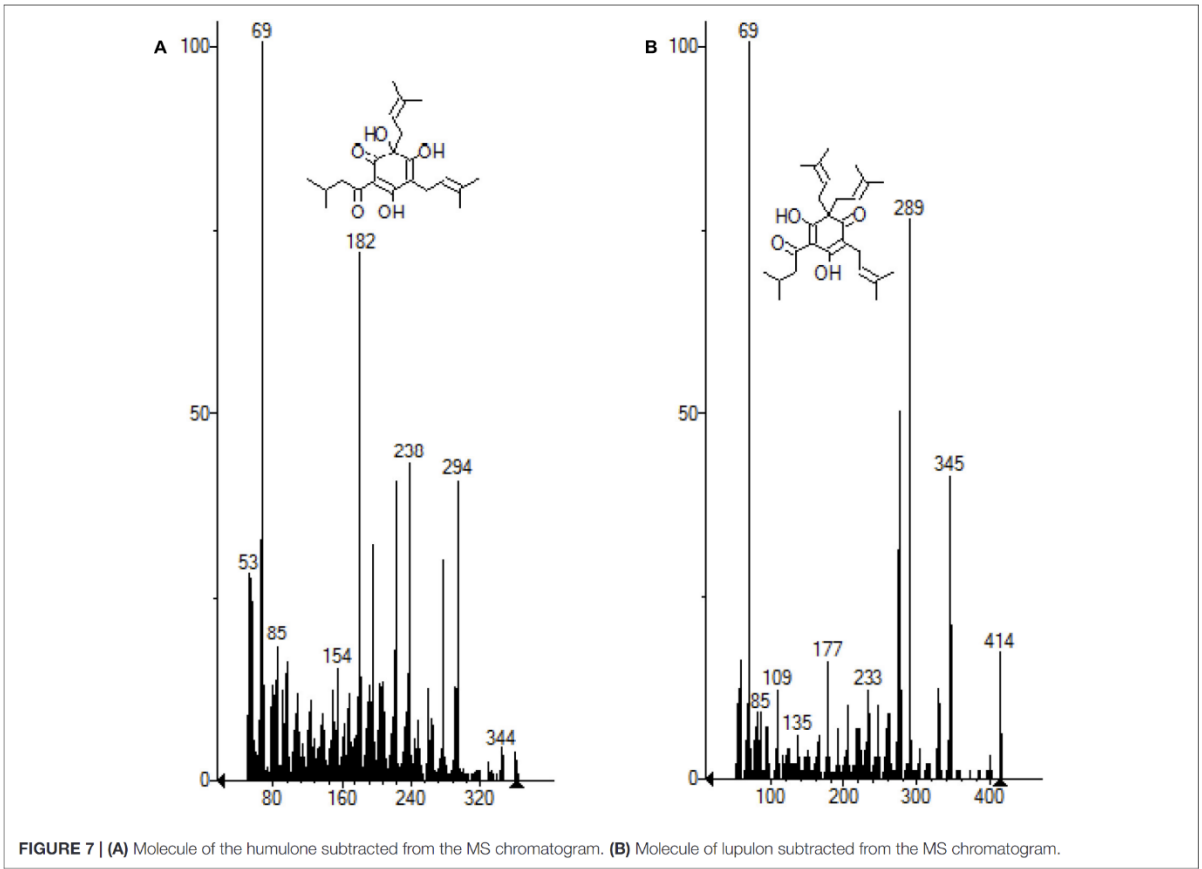


FIGURE 7 | (A) Molecule of the humulone subtracted from the MS chromatogram. **(B)** Molecule of lupulon subtracted from the MS chromatogram.

descriptors. They are also insoluble in wort and beer but their oxidation leads to derivative compounds, such as their epoxides (for example humulene epoxide) or humulol alcohols, which appear in the final product depending on the time of adding hops. As previously mentioned, sesquiterpenes such as humulene, caryophyllene and β-pinene (oxidation product of myrcene) are the main constituents of essential oil of

fresh hops. More specifically, it is evident **(Table 5)** that the application of PEF has a small but significant increase in the concentration of these compounds. In particular, in the control samples, the humulene, caryophyllene and β-pinene had a concentration of 0.89, 0.27, and 0.09 mg/L, while in the treated samples at PEF, there was an increase of 0.94, 0.29, and 0.08 mg/L, respectively. The application of PEF had a limited influence on the concentration of these volatile compounds and mainly in the monoterpens. The oxygenated fractions of the hop aroma (Deinzer and Yang, 1994) can be synergistic by contributing to the “hops” of beer (Siebert, 1994). All of these compounds have an active flavor in beer with very low flavor thresholds (ppb) and depending on when they are added, they play an important role in the character of hops (Preis and Mitter, 1995). From this point of view, even a slight increase in terpene precursors (humulene, caryophyllene and myrcene) is important for the final hoppy taste of beer.Liquid-liquid extraction involves a heating step which can degrade the initial profile of volatiles. The SPE method used before the GC-MS analysis allowed a clear separation of the compounds and a “clean” chromatogram, as shown in **Figure 6**.The molecules of the main acids (humulone and lupulone) of the MS chromatogram are presented in the **Figures 7A,B. Table 5** shows the effect of PEF on the (average) concentration of α and β-acids in bitter hop varieties. The concentration of acids should be higher in bitter hops. The extraction of α and β-acids soluble in methanol also increases with the application of PEF, with the intensity of the electric field and with the extension of the duration of the treatment.

TABLE 5 | Volatile analysis (mg/L) of bitter hop with or without PEF treatment.

Compound	Pef treated		Control	
	Average	S.D.	Average	S.D.
β-Myrcene	0.083	0.052	0.092	0.003
2-octanol	0.125	0.000	0.125	0.000
Caryophyllene	0.290	0.175	0.266	0.070
β-Cubebene	0.019	0.009	n.d.	
Humulene	0.943	0.578	0.889	0.203
γ-Muurolene	0.023	0.015	0.022	0.019
γ-Cadinene	0.032	0.004	0.023	0.006
β-Cadinene	0.008	0.002	n.d.	
α-Cadinene	0.008	0.005	0.008	0.003
δ-Cadinene	0.069	0.040	n.d.	
Geranyl isobutyrate	n.d.*		0.034	0.019
Hexadecane	0.036	0.008	0.023	0.002
Humulene epoxide 2	n.d.		0.025	0.006
Hexadecanoic acid methyl ester	0.053	0.021	0.047	0.010
Dehydro-cohumulinic acid or 3-Hydroxy-2-isobutyryl-5-(3-methyl-2-butenyl)-2,4-cyclopentadien-1-one	0.069	0.033	0.335	0.277
3-hydroxy-2-(1-hydroxy-3-methylbutylidene)-5-(3-methyl-2-butenylidene)-3-Cyclopenten-1-one	0.510	0.159	1.754	1.333
Linoleic acid methyl ester	0.036	0.019	0.032	0.006
Humulone	0.498	0.130	0.837	0.045
Isohumulone	5.977	1.134	4.325	0.275
Lupulone	16.630	2.498	11.269	0.926

**n.d. = Not detected.*

As indicated in the samples treated with PEF, the bitter acids in the sample had a concentration 40.66% higher than that of the control. More specifically, the samples treated with PEF had a higher concentration compared to the control (25.45 and 47.56% of α and β-acids, respectively). This result is of capital importance since the aim of bitter hops is to strengthen bitterness.

DPPH•

The treated extracts maintained their antiradical activity **(Table 7)** and, in the case of extracted methanol, an increase of about 10% was observed. The water treated samples demonstrated almost the same antiradical activity. The low water extractability probably does not allow the proper evaluation of the results.

CONCLUSION

In conclusion, this study aimed to extract α-acids and β-acids of two different varieties of hops using PEF. During these experiments, different solvents and different methods of analysis were used. According to the results, samples of hops treated with PEF showed higher concentrations of humulones and lupulones (the main representatives of α-acids and β-acids, respectively). PEF conditions (1.5 kV/cm; 15 μs and 1800 pulses) increased the total bitter acids (a β) and sesquiterpenes extraction from bitter hop approximatively by 1.3 times. The PEF treatment enhanced the extraction of α-acids from 21 to 100% and from 9 to 120% for β-acids. The amount of extracted acids was a function of the solvent and the time of treatment. PEF treatment of hop pellets did not cause any substantial changes in HSI that would indicate possible further degradation. Hops maintained their antiradical activity, which, in some cases, was increased. Therefore, it can be concluded that the extraction of α- and β-acids was enhanced by PEF application and should be further investigated in order to optimize their concentration by utilizing water base solvents or by minimizing the time of the PEF treatment in pilot plant conditions before industrial applications.

TABLE 6 | Averages (mg/L) of α- and β-acids of bitter hop PEF treated and control samples extracted with methanol.

Method	Acids	Pef Treated		Control		Increase (%)
		Average	S.D.	Average	S.D.	
GC-MS	α-acids	6.4	1.0	5.2	0.2	25.45
	β-acids	16.6	2.5	11.3	0.9	47.56
UV–vis	α-acids	16.2	1.0	13.1	0.1	23.66
	β-acids	9.6	2.4	8.8	0.2	9.09

TABLE 7 | Percentage of inhibition of DPPH• radical (I%) of hop extracts.

Sample	*I%*	
	PEF treated	Control
Hydrated (bitter)	70.27	69.89
Methanol (bitter)	73.19	83.35
Methanol (aromatic)	82.47	81.32

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

GN: conception and execution of the PEF work. ET: analysis of GC-MS. FD: analysis of hop and antioxidant properties. EB: writing – review and editing. SL, PT, and VD: materials and methods setup.

FUNDING

This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH – CREATE – INNOVATE (project code: T1EDK-03762).

REFERENCES

Alderton, G., Bailey, G. R., Lewis, J. C., and Stitt, F. (1954). Spectrophotometric determination of humulone complex and lupulone in hops. *Anal. Chem.* 26, 983–992. doi: 10.1021/ac60090a010

Alvarez, I., Pagan, R., Condon, S., and Raso, J. (2003). The influence of process parameters for the inactivation of Listeria monocytogenes by pulsed electric fields. *Intern. J. Food Microbiol.* 87, 87–95. doi: 10.1016/s0168-1605(03)00056-4 American Society of Brewing Chemists (1992). *Methods of Analysis*, 8th Edn, St.Paul, MN: ASBC.

Bimakr, M., Ganjloo, A., Zarringhalam, S., and Ansarian, E. (2017). Ultrasound- assisted extraction of bioactive compounds from *Malva sylvestris* leaves and its comparison with agitated bed extraction technique. *Food Sci. Biotechnol.* 26, 1481–1490. doi: 10.1007/s10068-017-0229-5

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature* 181, 1199–1200. doi: 10.1038/1811199a0

Boussetta, N., Lanoisellé, J.-L., Bedel-Cloutour, C., and Vorobiev, E. (2009). Extraction of soluble matter from grape pomace by high voltage electrical discharges for polyphenol recovery: effect of sulphur dioxide and thermal treatments. *J. Food Eng.* 95, 192–198. doi: 10.1016/j.jfoodeng.2009.04.030

Boussetta, N., Vorobiev, E., Deloison, V., Pochez, F., Falcimaigne-Cordin, A., and Lanoisellé, J.-L. (2011). Valorisation of grape pomace by the extraction of phenolic antioxidants: application of high voltage electrical discharges. *Food Chem.* 128, 364–370. doi: 10.1016/j.foodchem.2011.03.035

Bozinou, E., Karageorgou, I., Batra, G., Dourtoglou, V. G., and Lalas, S. I. (2019). Pulsed electric field extraction and antioxidant activity determination of *Moringa oleifera* dry leaves: *α* comparative study with other extraction techniques. *Beverages* 5:8. doi: 10.3390/beverages5010008

Cukjati, D., Batiuskaite, D., André, F., Miklavcic, D., and Mir, L. M. (2007). Real time electroporation control for accurate and safe in vivo non-viral gene therapy. Bioelectrochemistry 70, 501–507. doi: 10.1016/j.bioelechem.2006.11.001

Deinzer, M., and Yang, X. (1994). Hop aroma: character impact compounds found in beer, methods of formation of individual compounds. Proc. Eur. Brew. Convent. 22, 1–17.

Delsart, C., Ghidossi, R., Poupot, C., Cholet, C., Grimi, N., and Vorobiev, E. (2012). Enhanced extraction of phenolic compounds from merlot grapes by pulsed electric field treatment. Am. J. Enol. Viticult. 63:2. doi: 10.5344/ajev.2012.11088 Delsart, C., Grimi, N., Boussetta, N., Miot Sertier, C., Ghidossi, R., Vorobiev, E., et al. (2015). Impact of pulsed-electric field and high-voltage electrical discharges on red wine microbial stabilization and quality characteristics.

J. Appl. Microbiol. I 120, 152–164. doi: 10.1111/jam.12981

Dieckmann, R. H., and Palamand, R. S. (1974). Autoxidation of some constituents of hops. i. monoterpene hydrocarbon myrcene. J. Agr. Food Chem. 22, 498–503. doi: 10.1021/jf60193a033

Drosou, F., Yang, E., Marinea, M., Dourtoglou, E. G., Chatzilazarou, A., and Dourtoglou, V. G. (2017). “An assessment of potential applications with pulsed electric field in wines,” in Proceeding of the 40th World Congress of Vine and Wine, Sofia.

Egts, H., Durben, D. J., Dixson, J. A., and Zehfus, M. H. (2012). A multicomponent UV analysis of α- and β-acids in hops. J. Chem. Educ. 89, 117–120. doi: 10.1021/ed1010536

Eri, S., Khoo, B. K., Lech, J., and Hartmann, T. G. (2000). Direct thermal desorption-gas chromatography and gas chromatography-mass spectrometry profiling of hop (Humulus lupulus L.) essential oils in support of varietal characterization. J. Agric. Food Chem. 48, 1140–1149. doi: 10.1021/jf9911850

European Brewery Convention (2006). Analytica-EBC; Fachverlag Hans Carl, 2006; Section 7 Hops, Methods 7.2, 7.10 and 7.12. Nuernberg: EBC.

Eyres, G. T., and Dufour, J. P. (2009). “Hop essential oil: analysis, chemical composition and odor characteristics,” in Beer in Health and Disease Prevention, ed. V. R. Preedy (Cambridge, MA: Academic Press), 239–254. doi: 10.1016/ B978-0-12-373891-2.00022-5

Grahl, T., and Markl, H. (1996). Killing of microorganisms by pulsed electric fields.

Appl. Microbiol. Biotechnol. 45, 148–157. doi: 10.1007/s002530050663

Grimi, N., Lebovka, N. I., Vorobiev, E., and Vaxelaire, J. (2009). Effect of a pulsed electric field treatment on expression behavior and juice quality of chardonnay grape. Food Biophys. 4, 191–198. doi: 10.1007/s11483-009-9117-8

Grimi, N., Mamouni, F., Lebovka, N., Vorobiev, E., and Vaxelaire, J. (2011). Impact of apple processing modes on extracted juice quality: pressing assisted by pulsed electric fields. J. Food Eng. 103, 52–61. doi: 10.1016/j.jfoodeng.2010.09.019

Gyawali, R., Seo, H. Y., Lee, H. J., Song, H. P., Kim, D. H., Byun, M. W., et al. (2006). Effect of g-irradiation on volatile compounds of dried Welsh onion (Allium fistulosum L.). Radiat. Phys. Chem. 75, 322–328. doi: 10.1016/j.radphyschem. 2005.07.001

Heinz, V., Toepfl, S., and Knorr, D. (2003). Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment. Innov. Food Sci. Emerg. Technol. 4, 167–175. doi: 10.1016/s1466-8564(03) 00017-1

Hossain, M. B., Tiwari, B. K., Gangopadhyay, N., O’Donnell, C. P., Brunton, N. P., and Rai, D. K. (2014). Ultrasonic extraction of steroidal alkaloids from potato peel waste. Ultrason. Sonochem. 21, 1470–1476. doi: 10.1016/j.ultsonch.2014. 01.023

Kunze, W. (2004). Technology Brewing and Malting. Berlin: VLB.

Maged, E. A. M., and Ayman, H. A. E. (2012). Pulsed Electric Fields for Food Processing Technology. London: IntechOpen.

Nance, M., Setzer, R., and William, N. (2011). Volatile components of aroma hops (Humulus lupulus L.) commonly used in beer brewing. J. Brew. Distill. 2, 16–22.

Nickerson, G. B., and Van Engel, L. (1992). Hop aroma component profile and the aroma unit. J. Am. Soc. Brew. Chem. 50, 77–81. doi: 10.1094/asbcj-50-0077 Ohshima, T., and Sato, M. (2004). Bacterial sterilization and intracellular protein release by a pulsed electric field. Adv. Biochem. Engin. Biotechnol. 90, 113–133. doi: 10.1007/b94194

Peacock, V. E., and Deinzer, M. L. (1981). Chemistry of hop aroma in beer. J. Am.Soc. Brew. Chem. 39, 136–141. doi: 10.1094/asbcj-39-0136

Peacock, V. E., and McCarty, P. (1992). Varietal identification of hops and hop pellets. Techn. Q. Master Brew. Assoc. Am. 29, 81–85.

Pinela, J., Antonio, A. L., Cabo Verde, S., Barreira, J. C. M., Carvalho, A. M., Oliveira, M. B. P. P., et al. (2014). “Effects of gamma irradiation and extraction method in the antioxidant potential of wild and commercial Tuberaria lignosa samples,” in Proceedings of the International Symposium on Food Safety and Quality: Applications of Nuclear and Related Techniques - IAEA CN-222At, Viena.

Preis, F., and Mitter, W. (1995). The rediscovery of first wort hopping. Brauw.Intern. 13, 308–315.

Rettberg, N., Biendl, M., and Garbe, L. A. (2018). Hop aroma and hoppy beer flavor: chemical backgrounds and analytical tools: a review. J. Am. Soc. Brew. Chem. 76, 1–20. doi: 10.1080/03610470.2017.1402574

Roberts, T. R. (2016). “Hops,” in Brewing Materials and Processes: A Practical Approach to Beer Excellence, ed. C. W. Bamforth (Cambridge, MA: Academic Press), 47–75. doi: 10.1016/C2013-0-13349-1

Siebert, K. J. (1994). “Sensory analysis of hop oil-derived compounds in beer; flavor effects of individual compounds. Quality control,” in Proceedings of the Monograph XXII E.B.C. Symposium on Hops. European Brewery Convention, Zoeterwoude.

Steinhaus, M., and Schieberle, P. (2000). Comparison of the most odor active compounds in fresh and dried hop cones (Humulus lupulus L. variety spalter select) based on GC-olfactometry and odor dilution techniques. J. Agr. Food Chem. 48, 1776–1783. doi: 10.1021/jf990514l

Stevens, R. (1967). The chemistry of hop constituents. Chem. Rev. Am. Soc. 67, 19–71. doi: 10.1021/cr60245a002

Touya, G. (2005). Contribution à l’étude Expérimentale des Décharges Électriques Dans L’eau Et des Ondes De Pression Associées. Réalisation d’un Prototype Industriel 100 Kj Pour le Traitement de Déchets Par Puissances Électriques Pulsées. Ph.D. thesis, Université de Pau et des Pays de l’Adour, Pau.

Van Holle, A., Van Landschoot, A., Roldán-Ruiz, I., Naudts, D., and Keukeleire,

D. (2017). The brewing value of amarillo hops (Humulus lupulus L.) grown in northwestern USA: a preliminary study of terroir significance. Instit. Brew. Distil. 123, 312–318. doi: 10.1002/jib.433

Vorobiev, E., and Lebovka, N. (2010). Enhanced extraction from solid foods and biosuspensions by pulsed electrical energy. Food Eng. Rev. 2, 95–108. doi: 10.1007/s12393-010-9021-5

Zeng, X. A., Yu, S. J., Zhang, L., and Chen, X. D. (2008). The effects of AC electric field on wine maturation. Innov. Food Sci. Emerg. Technol. 9, 463–468. doi: 10.1016/j.ifset.2008.03.002

Zhang, B., Zeng, X. A., Sun, D., Yu, S. J., Yang, M. F., and Ma, S. (2012). Effect of electric field treatments on brandy aging in oak barrels. Food Bioprocess Technol. 6, 1635–1643. doi: 10.1007/s11947-012-0788-7

Zimmermann, U. (1986). Electrical breakdown, electropermeabilization and electrofusion. Rev. Physiol. Biochem. Pharmacol. 105, 175–256. doi: 10.1007/ bfb0034499

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Δημοσίευση ΙΙ

In situ Creation of the Natural Phenolic Aromas of Beer: A Pulsed Electric Field Applied to Wort-Enriched Flax Seeds

Evangelia A. Tsapou[1], George Ntourtoglou[1], Fotini Drosou[1], Panagiotis Tataridis[1], Thalia Dourtoglou[1], Stavros Lalas[2] and Vassilis Dourtoglou[1]*

1 **Department of Wine, Vine, and Beverage Sciences, School of Food Science, University of West Attica, Athens, Greece**
2 **Department of Food Science and Nutrition, University of Thessaly, Karditsa, Greece**

ABSTRACT

To fine tune the production of phenolic aromas in beer, a pulsed electric field (PEF) was applied to beer wort, which was enriched with flax seeds. The choice of flax seeds as a source of FA is based on its high content of ferulic precursors and their intrinsic nutritional value. PEF was applied to ground flax seeds, with and without beta glycosidase. Fermentation was carried out with Saccharomyces and non-Saccharomyces yeast strains. Moreover, 4-vinylguaiacol (4-VG), a flavor highly active derived from volatile phenol, was produced by decarboxylation of ferulic acid (FA), or its precursor and flavor inactive (4-hydroxy-3-methoxycinnamic acid). All yeast strains could metabolize FA into 4-VG, using the pure compound in the synthetic medium or in flax seeds, with the best quantity produced by *Saccharomyces cerevisiae* as a precursor. The method yields 4-VG production efficiencies up to 120% (mgL−1). Experimental treatment conditions were conducted with E= 1 kV/cm, total time treatment 15 min (peak time ti = 1 μs, pause time tp = 1 ms, Total pulses 9003). Treatment efficacy is independent of the fermentation yeast.

Keywords: ferulic acid, flax seeds, pulsed electric field (PEF), 4-vinylguaiacol (4-VG), *Saccharomyces cerevisiae*, non-Saccharomyces, yeast strain

INTRODUCTION

Flavoring with natural compounds is well-known in the food and beverage industry, driven by consistent consumer demand for more “natural” foods. Industries can improve the techniques of extraction or isolation of aromatic compounds by frequently applying two or more technologies to isolate target molecules, thereby rendering the final aroma more attractive. However, the most difficult issue is to empower the typical aromatic

character without changing the final product (from the commonly accepted taste and aroma). This is done by hydrolyzing enzymatically bound aromatic compounds in polymeric precursors with glycolytic enzymes (i.e., in wine), by adding aromatic compound precursors or using physicochemical methods (e.g., distillation and enrichment of the beverage with the heads of distillates).

Beer as a complex beverage contains many flavor active compounds, as brewers try to reach an appropriate balance of desired aromas and to avoid off-flavors. Phenolic compounds are always present in the final product; they can be extracted from grains and hops during the process of mashing or brewing. Some phenolic compounds have a small impact on beer, while others may cause some desirable or undesirable effects (Lentz, 2018).

Moreover, 4-vinylguaiacol (4-VG) is an important constituent in bier (Tressl et al., 1976), distilled spirits (whiskey, rum) (Lee et al., 2000), contributing to the overall sweet smoky background. Found in tobacco, roasted peanuts, traminer wines is considered to be an important aroma contributor. Conventional *Saccharomyces cerevisiae* brewing yeasts ferment beers, in which a clove-like aroma is desired (Vanbeneden and Gils, 2008; Goncalves et al., 2016). Despite being undesirable in bottom-fermented beers, 4-VG is a well-known, positive aroma compound found in top-fermented beers, including those brewed with unmalted wheat for Belgian white beers, as well as those brewed with malted wheat for German Weizen and Rauch beers. However, in most of the top-fermented blond and dark beers, this volatile compound's presence is essential for overall flavor perception (Goncalves et al., 2016). Hence, it is important for flavor perception in the best beers (Zhu and Cui, 2013; Mertens et al., 2017).

Ferulic acid (FA) is the precursor of 4-VG and is found in plant cell walls in its free form or is covalently bonded to the biopolymers (Johnson et al., 2000; Strandås et al., 2008; Tanruean and Rakariyatham, 2016).

During this work we attempt to reach phenolic flavor enhancement, avoiding off-flavors by a combination of two technologies applied to beer wort-enriched with flax seeds. The target compound was 4-VG. These applied technologies are summarized below:

- In addition to the wort, rich FA derivative precursors (contained in flax seeds) and hydrolysis of the covalently bonded precursor from natural polymers was handled by the application of β-glucosidase from almonds, with a wide substrate spectrum of C5 and C6 monosaccharides and oligosaccharides. The industrial extension would be by applying a β-glycosidase from a microbial source.
- Extraction of the precursor, assisted by PEF.

PEF is a relatively new technique and when applied for extraction, it disorganizes the plant or microbial cell by disrupting membranes and releasing cell metabolites from the

inner to outer part of the cell (Yang et al., 2016). The induced electric fields destroy the membrane of microbial or plant cells, thus leading to complex phenomena from cell restructuration to cell death (Zeng et al., 2008; Delsart et al., 2012; Zhang et al., 2012; Bozinou et al., 2019). PEF has been applied to other crops of industrial interest, such as grapes, onions, potatoes, etc. It was mainly used as a non- thermal treatment of liquid foods, to inactivate microorganisms (Grahl and Markl, 1996; Alvarez et al., 2003). Other researchers introduced electric field treatment to accelerate the aging of young wine, due to extraction of flavor compounds from wood (Zeng et al., 2008; Drosou et al., 2017) or to improve the phenolic recovery, as extraction pre –treatment in beer (Martin-Garcia et al., 2020). To increase the amount of FA in the final product, a combination of a natural precursor (raw material) (rich in FA, such as flax seed), and an enzymatic and electrotechnique were used before fermentation. To demonstrate the versatility of the technique, four strains of yeast were used: *S. cerevisiae* and three other non-Saccharomyces. The technique was applied in a synthetic medium or in beer wort.

TABLE 1 | 4VG formation by yeast strains cultivated in synthetic Medium A with and without the presence of FA, or the presence of flax seeds.

4VG	Medium A without FA		Medium A + 48 mg/L of FA		Medium A + 26,7 g/L of flax seeds	
	Average	Efficacy [%]	Average	Efficacy [%]	Gold	Brown
S.C.	n.d.*	n.d	31.81 ± 14.85	66.27	0.732	0.077
Prelude	n.d	n.d	11.38 ± 6.70	23.71	0.086	0.079
Biodiva	n.d	n.d	14.91 ± 4.83	31.06	0.083	0.000
Mets	n.d	n.d	22.04 ± 9.50	45.92	0.285	0.003

*n.d, not detected.

TABLE 2 | Results of control and PEF fermentations with brown and gold flax seeds in beer wort: Concentration of 4VG (mgL^{-1}) with the presence of β-glycosidase (+) and absence (-).

4VG		Brown flax seed		Gold flax seed		*t*-test
		PEF	Control	PEF	Control	
Mets	(–)	10.09 ± 2.11	8.44	13.99 ± 2.74	3.12	0.22
	(+)	11.49 ± 2.76	12.58	15.81 ± 3.52	5.62	
Prelude	(-)	10.17 ± 1.99	6.26	10.78 ± 2.05	16.08	0.24
	(+)	11.45 ± 1.14	6.58	16.66 ± 3.73	15.73	
Biodiva	(-)	10.81 ± 1.82	4.86	10.09 ± 0.45	9.34	0.23
	(+)	16.06 ± 4.31	3.52	10.92 ± 1.99	9.73	
S.C.	(–)	11.90 ± 0.65	5.91	11.83 ± 2.45	6.94	0.46
	(+)	9.19 ± 0.56	4.54	15.63 ± 3.83	7.88	

TABLE 3 | Concentration of 4VG (averages and SD) in mgL^{-1} without the presence of β-glycosidase.

4VG	Brown flax seed					
	Control		Average	PEF		Average
Mets	8.44	12.58	10.51 ± 2.93	10.09	11.49	10.79 ± 0.98
Prelude	6.26	6.58	6.42 ± 0.22	10.17	11.45	10.81 ± 0.90
Biodiva	4.86	3.52	4.19 ± 0.95	10.81	16.06	13.43 ± 3.71
S.C.	5.91	4.54	5.22 ± 0.97	11.90	9.19	10.55 ± 1.92
	Gold flax seed					
	Control		Average	PEF		Average
Mets	3.12	5.62	4.37 ± 1.77	13.99	15.81	14.90 ± 1.29
Prelude	16.08	15.73	15.90 ± 0.25	10.78	16.66	13.72 ± 4.16
Biodiva	9.34	9.73	9.53 ± 0.28	10.09	10.92	10.50 ± 0.58
S.C.	6.94	7.88	7.41 ± 0.67	11.83	15.63	13.73 ± 2.69

MATERIALS AND METHODS

Chemicals

Dichloromethane, chloroform, methanol, pentane (95%), diethyl ether (95%), and anhydrous sodium sulfate were purchased from Chem Lab (Athens, Greece). All reagents were of analytical quality: β-glycosidase and 3-octanol were used as an internal standard, while all chemicals used for medium A were purchased from Sigma Aldrich (St. Louis, MO, USA).

Fermentation

• Yeast strains

Four yeasts strains (*S. cerevisiae* and three non-Saccharomyces yeast strains) were used in a concentration of 100 mgL−1 for each fermentation: *S. cerevisiae* US-05 (Fermentis), (SC) *Toluraspora delbrueckii* Prelude (Hansen), (Prelude) *Toluraspora*

delbrueckii Biodiva 291 (Biodiva) (Lallemand), and *Metschnikowia pulcherrima* (Lallemand) (Mets).

• Batch of synthetic medium A supplied with pure FA

Synthetic medium A for main cultures, inoculated with 0.1 gL−1 lyophilized microorganisms, was prepared by dissolving KH2PO4 1 gL−1; K2HPO4 1 gL−1 (NH4)2SO4 2 gL−1; MgSO4·7H2O 0.2 gL−1; ZnSO4·5H2O 0.2 gL−1; glucose 9 gL−1; maltose 86 gL−1; fructose 5 gL−1; (pH 5). Media were prepared in deionized water that day and autoclaved.

For media containing FA, pure FA is added to each sample at 12 mg for 150 mL (48 mgL−1). The volume of each fermentation was 150 mL for each yeast strain. Samples were kept at room temperature for 7 days to conduct yeast fermentation. All fermentations were performed in duplicate.

• Batch in Synthetic medium A supplied with flax seeds

Both varieties of flax seeds (gold and brown) were purchased from a local Greek market (Athens, Greece). Two fermentation bottles (150 mL) with cells were cultured batch-wise on synthetic medium A for each mentioned yeast strain. Both varieties of flax seeds (brown and gold) ground in a plate mill were added to the bottled after being crushed, for a quantity of 4 g per bottle. As such, 625 µL of a prepared solution of 4 gL−1 β-glucosidase from almonds (lyophilized powder, ≥ 2 units/mg solid) at Ph 7.4 was added to each vial. This was left for 30 min for enzyme activation. As stated, all samples were incubated at 35◦C for 7 days and conducted in duplicate.

• Flax seeds added to wort for beer production, plus PEF and Control fermentations, with and without β-glucosidase from almonds

Wort production:

➢ Mashing process: 1 kg of Pils malt (Macedonian Thrace Brewery S.A., Athens, Greece) ground in 1–1.2 mm and mixed with water at 55◦C. The program was: 1◦C min−1 up to 63◦C to remain for 1 h. We find 1◦C min−1 up to 72◦C, remaining for approximately 15 min, and 1◦C min−1 up to 78◦C for 5 min. The process of brewing in a bag (BIAB) was used, and after the mash rest, the bag was attached to drain above the kettle and then squeezed.

➢ Sparging step: 1 L of water at 77–80◦C was used for sparging during the mash rest, and again after squeezing the grain bag.

➢ Boiling process: The last bag was boiled for 90 min and the wort was used under sterilized conditions without adding hops. Wort was added in sterilized fermentable

bottles (48 in total) at 60 mL, with 1.6 g of each variety of flax seed added to the bottles (16 with gold flax and 16 with brown flax).

PEF was applied in 16 substrates in duplicate (8 worts with gold flax and 8 worts with brown flax). The rest of the bottles (8 with gold flax and 8 with brown flax) were used as Control substrates without PEF treatment.

All substrates were inoculated with the same four yeast strains. At that point, 250 μL of the prepared solution of β-glucosidase was added to a bottle of each yeast and the other was used as the Control enzyme and for PEF fermentation. All samples were incubated at 35◦C for 7 days.

Pulsed Electric Field Procedure

The PEF equipment used was described previously by Ntourtoglou et al. (2020). The treatment chamber (TC) consisted of two rectangular flat stainless steel electrodes measuring 10 by 10 cm in size, separated by Teflon bars. The distance between the two electrodes was 1 cm. The electric field strength E was evaluated as E = U/d, where “U” is the applied voltage and “d” is the distance between two electrodes (d = 10 mm). In each case,

treatment was calculated as: [t = (ti + tp) × P], ti = peak time duration (μs), tp = pause time (ms) and P = number of pulses. Experimental treatment conditions were conducted with E= 1 kV/cm, t = 15 min (ti = 1μs, tp = 1 ms, 9003 pulses).

Sample Preparation for GC-MS

In addition, 40 mL of each sample was mixed with organic solvents (mixture of 20 mL of pentane and 20 mL of diethyl ether) for 10 min at room temperature. The samples were centrifuged at 3,500 rpm for 10 min to separate the phases (Hermle Z200A, Milan, Italy). The supernatant was extracted a second time, using the same volume of solvent for 10 min. The organic layer was washed with distilled water in a separation funnel, while the organic phase was dried over anhydrous sodium sulfate and filtered. Samples were condensed in a flash evaporator and compressed with nitrogen until they were dry weight. Finally, 100 μL of dichloromethane was added to samples, from which1.0 μL was used for GC–MS analysis. A concentration of 4-VG was calculated using a standard curve for Control and PEF fermentation, while the ones with synthetic medium 10 μL of 3-octanol (2,500 ppm) were added as an internal standard after being filtered.

Gas Chromatography-Mass Spectrometry Analysis

Each sample was subjected to GC, coupled with MS analysis, using an Agilent 6890 series GC System (Wilmington, DE, USA), described by Ntourtoglou et al. (2020). All data were recorded with the Turbomass 5.0 ChemStation software (Agilent).

Statistical Analysis

Statistical analysis for the standard deviation (SD) of the means and t-tests were carried out with Excel 2013 (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Experiments in the Presence of Free FA or Flax Seeds: A Source of FA in Synthetic Medium A

As shown in **Table 1**, none of the four yeast strains was capable of producing 4-VG without the external addition of free FA or flax seeds to the synthetic medium A. When free FA was included in medium A, all strains of yeast demonstrated biotransformation of FA into 4-VG.

Three important conclusions are based on the findings:

1. Saccharomyces and non-Saccharomyces are strains with high exhibited FA decarboxylase activity. *S. cerevisiae* brewing yeasts contain active ferulate decarboxylase enzymes that can transform trans-FA into 4-VG. The enzyme responsible for the decarboxylation of FA is FDC1 (FA decarboxylase) (Goncalves et al., 2016). This can be seen in **Table 1**.

2. The optimum temperature, pH, and sugar content for biotransformation was: glucose 9 gL−1; maltose 86 gL−1; fructose 5 gL−1; (pH 5) at 35◦C. Since FA, is the starting material in the synthesis of vanillin and other aromatic compounds, such as vanillic acid, vanillin, and vanillic alcohol (Kumar and Pruthi, 2014; Tanruean and Rakariyatham, 2016), we have tried to find these substances in our experiments but without success. Probably the enzymes for the subsequent biotransformation of 4-VG to vanillin were absent.

3. The highest conversion of pure FA to 4-VG was obtained by SC (66.27%), while Prelude had the lowest (23.71%).

This is in line with the conclusion of Watanabe et al. (2009).

When flax seeds were added to medium A, 4-VG is produced. In these fermentations, β-glucosidase was added. With this, the highest amount of 4-VG was produced by SC

when golden flax seeds (0.732 mgL−1) were used, while the non-Saccharomyces spp. produced considerably lower amounts (0.003–0.285 mgL−1).

The Effect of Flax Seeds and PEF in Beer Wort

Table 2 shows the results from the Control and PEF fermentations, while β-glycosidase was added again. An increase in the concentration of 4-VG was also observed. All yeasts proved capable of producing 4-VG, when FA was available from wort and flax seeds during fermentation: in this process, the first condition is the presence of the enzyme, which is added to liberate from the β-glucosidic form the covalently bonded forms of FA derivatives, or to neutralize the very tiny amounts of cyanate ions from flax seeds. Given independent-samples, the one-tailed t-test was conducted to compare the concentration of 4-VG both with the enzyme and without it during fermentation. The only factor that was checked during the t-test was the enzyme, so the results concern Control and PEF fermentations, and each variety of flax seeds. There was not a significant difference in the concentrations of 4-VG for any yeast. The results of the t-test are presented in **Table 2**. Since there is no significant difference concerning the

presence of the enzyme, averages of the fermentations with and without β-glycosidase are shown for each yeast in **Table 3**. In this table, it is obvious that PEF treatment in the substrates before fermentation resulted in an increased production yield of 4-VG, by an average of 120%. Specifically, to evaluate the differences due to PEF, the average PEF fermentations compared to Control fermentations and the results are presented in **Figures 1A,B**. It appears that the gold flax variety produces better results in 4-VG from PEF fermentations. For yeasts, SC had more stable results and presented a difference between Control and PEF fermentations for flax seed varieties. Independent-samples and a one-tailed t-test were performed, without considering yeast strains for assessing the statistical difference of the concentration of 4-VG, with and without PEF treatment in the substrates. The results show a significant difference ($p = 0.0005$). When the external electric field is applied to the must enriched in flax, a critical electric potential across the cell membrane is induced. This potential causes a profound modification of the cell

membrane which is rather mechanical. Consequently, the permeability of the membrane increases considerably, pores are formed simultaneously and release FA derivatives which are localized to the internal part of the cell. In our case, the disorganization occurs on the wort and on the flax seed. The FA derivatives have further been

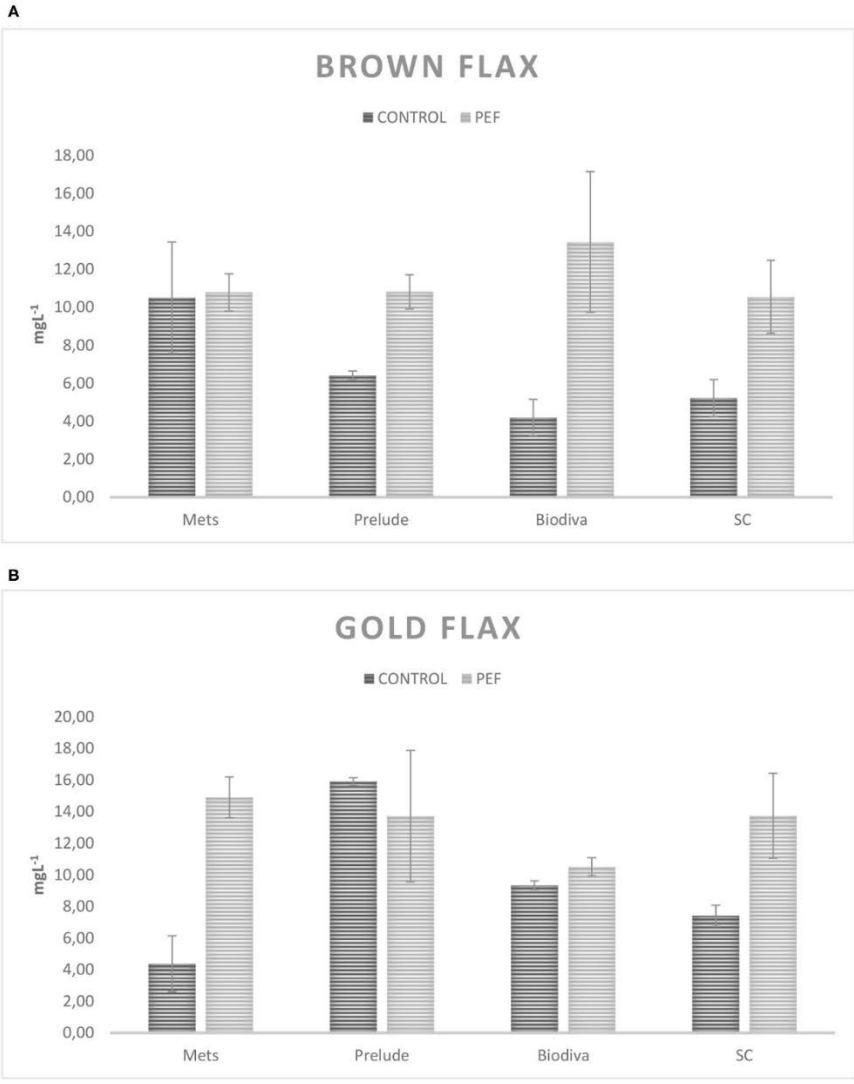


FIGURE 1 | Differences in the concentration of 4VG (mgL^{-1}) among PEF fermentations and control: **(A)** brown flax seed variety, **(B)** gold flax seed variety. Error bars represent SD.

decarboxylated from yeast enzymes to yielding higher amounts of 4-VG.

ADVANTAGES OF THE METHOD

Modulation of the Phenolic Flavor

There are many influences on the formation of phenols in beer: the proportion of wheat malt in the grist, the mashing conditions, and wort boiling, as well as the fermentation procedure, yeast strain, and contamination presence (Bartolome et al., 1996). Decarboxylation of flavor-inactive phenolic acids with a high flavor threshold like FA in 4-VG was viable until now in two ways: high temperature treatment during the beer production process or by enzymatic decarboxylation in fermentations (McMurrough et al., 1996). Both, however, present disadvantages. With the proposed method, the phenolic flavor can be modulated by increasing or decreasing the time of PEF treatment as in the case of extraction of alpha acids from hops (Ntourtoglou et al., 2020), or by controlling the quantity of flax seeds.

Modulating the phenolic flavor serves another purpose. An equilibrium must be respected between enhancement of aroma complexity and its phenolic off-flavor character. Indeed, 4-VG is an ingredient of phenolic off-flavors (Zhu and Cui, 2013),

which are possibly one of the unwanted compounds in beer production. Phenolic off-flavors have a low flavor threshold (0.2–0.4 mgL−1) and are characterized by a clove and medical aroma,highly undesirable in most beers (Mertens et al., 2017). Yet it is essential for the flavor of some wheat beer styles; *S. cerevisiae* brewing yeasts are used in the fermentation of beers in which a clove-like aroma is actually desired (Vanbeneden and Gils, 2008; Goncalves et al., 2016). The choice of the yeast, the conditions of PEF treatment, the quantity of flax seeds, are parameters of this method easily adapted to each beer style.

Enhancement of Antioxidant Capacity

Naturally occurring phenols in malt (germinated barley) have proven their antioxidant activities. Although FA is potentially a good antioxidant of beer, its action is limited due to low concentration in free form. Any pathway or physicochemical method that involves hydrolysis of cell wall feruloylated polysaccharides to the free form of FA during mashing and kilning is suitable to improve the natural antioxidant capacity of the wort, as well as the beer obtained from it. From this view the proposed technology enhances the antioxidant capacity of the wort or the beer.

Extension of the Work to Other Cell-Wall Degrading Enzymes

FA is mainly esterified to arabinofuranosyl residues of heteroxylans in barley cell walls (Mathew and Abraham, 2006). FA esterases can release FA from feruloylated plant cell wall polysaccharides, enhanced by the presence of cell-wall degrading enzymes (Bartolome et al., 1996). Research shows that when mashing, decarboxylation of FA is optimal.

Attempts have been made to obtain FA enzymatically, which is difficult due to covalent bonds between FA and biopolymers in plant cell walls (Fincher and Stone, 1986). It has been shown that enzymes from Streptomyces, e.g., acetyl xylan esterase and other xylanases, are used for enzymatic production of FA from defatted rice bran, suggesting extraction of FA from sources like corncob, raw rice bran, or wheat bran.

Disorganization of the plant cell wall using PEF will provide access to enzymes to target substrates releasing precursors convertible into desired compounds and enhancing the bio-flavoring.

CONCLUSIONS

In 15 min, PEF treatment of beer wort, supplemented with flax seeds before fermentation, yields 4-VG production efficiencies up to 120% (mgL−1). The wort was supplemented with a β-glycosidase, which is positive in terms of productivity in combination with PEF. The treatment chamber is box type, composed of two

rectangular parallel stainless steel electrodes. Voltage applied is 1 kV with pulses at 900 × 103. Treatment efficacy is independent of the fermentation yeast. In our work, four commercial strains of non-Saccharomyces and one Saccharomyces were used.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ supplementary material.

AUTHOR CONTRIBUTIONS

ET: main experiment, analysis of volatile compounds, writing, and editing the body text. GN: pulsed electric field design, construction, and main experiment. FD: analysis of volatile compounds and fermentations. PT: microorganisms culture and editing the body text. SL: editing the text and pulsed electric fields. TD: chemical analysis of volatile compounds. VD: designed the study and editing the body text. All authors contributed to the article and approved the submitted version.

REFERENCES

Alvarez, I., Pagan, R., Condon, S., and Raso, J. (2003). The influence of process parameters for the inactivation of listeria monocytogenes by pulsed electric fields. Int. J. Food Microbiol. 87, 87–95. doi: 10.1016/S0168-1605(03)00056-4

Bartolome, B., Garcia-Conesa, M. T., and Williamson, G. (1996). Release of the bioactive compound, ferulic acid, from malt extracts. Biochem. Soc. Trans. 24:379. doi: 10.1042/bst024379s

Bozinou, E., Karageorgou, I., Batra, G., Dourtoglou, V. G., and Lalas, S. I. (2019). Pulsed Electric Field extraction and antioxidant activity determination of Moringa oleifera dry leaves: a comparative study with other extraction techniques. Beverages 5:8. doi: 10.3390/beverages5010008

Delsart, C., Ghidossi, R., Poupot, C., Cholet, C., Grimi, N., Vorobiev, E., et al. (2012). Enhanced extraction of phenolic compounds from merlot grapes by pulsed electric field treatment. Am. J. Enol. Vitic. 63, 205–211. doi: 10.5344/ajev.2012.11088

Drosou, F., Yang, E., Marinea, M., Dourtoglou, E. G., Chatzilazarou, A., and Dourtoglou, V. G. (2017). An assessment of potential applications with pulsed electric field in wines. BIO Web Conf. 9:02010. doi: 10.1051/bioconf/20170902010

Fincher, G. B., and Stone, B. A. (1986). “Cell walls and their components in cereal grain processing,” in Advances in Cereal Science and Technology. ed Y. Pomeranz (St. Paul: American Association of Cereal Chemistry), 207–296.

Goncalves, M., Pontes, A., and Almeida, P. (2016). Distinct domestication trajectories in top-fermenting beer yeasts and wine yeasts. Curr. Biol. 26, 2750–2761. doi: 10.1016/j.cub.2016.08.040

Grahl, T., and Markl, H. (1996). Killing of microorganisms by pulsed electric fields.

Appl. Microbiol. Biotechnol. 45, 148–157 doi: 10.1007/s002530050663

Johnson, P., Kamal-Eldin, A., Lundgren, L. N., and Åman, P. (2000). HPLC method for analysis of secoisolariciresinol diglucoside in flax seeds. J. Agric. Food Chem. 48, 5216–5219. doi: 10.1021/jf0005871

Kumar, N., and Pruthi, V. (2014). Potential applications of ferulic acid from natural sources. Biotechnol. Rep. 4, 86–93. doi: 10.1016/j.btre.2014.09.002

Lee, K. Y. M., Paterson, A., Piggott, J. R., and Richardson, G. D. (2000). Perception of whisky flavour reference compounds by Scottish distillers. J. Inst. Brew. 106, 203–208. doi: 10.1002/j.2050-0416.2000.tb00058.x

Lentz, M. (2018). The impact of simple phenolic compounds on beer aroma and flavor. Fermentation 4:20. doi: 10.3390/fermentation4010020

Martin-Garcia, B., Tylewicz, U., Verardo, V., Pasini, F., Gomez-Caravaca, A. M., Caboni, M. F., et al. (2020). Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers’ spent grains. Innovat. Food Sci. Emerg. Technol. 64:102402. doi: 10.1016/j.ifset.2020.102402

Mathew, S., and Abraham, T. E. (2006). Bioconversions of ferulic acid, an hydroxycinnamic acid. Crit. Rev. Microbiol. 32,115–125. doi: 10.1080/10408410600709628

McMurrough, I., Madigan, D., and Donnelly, D. (1996). Control of ferulic acid and 4-vinyl guaiacol in brewing. J. Inst. Brew. 102, 327–332. doi: 10.1002/j.2050-0416.1996.tb00918.x

Mertens, S., Steensels, J., and Gallone, B. (2017). Rapid screening method for phenolic off-flavor (POF) production in yeast. J. Am. Soc. Brew. Chem. 75, 318–323. doi: 10.1094/ASBCJ-2017-4142-01

Ntourtoglou, G., Tsapou, E. A., Drosou, F., Bozinou, E., Lalas, S., Tataridis, P., et al. (2020). Pulsed electric field extraction of α and β-acids from pellets of Humulus Lupulus

(Hop). Front. Bioeng. Biotechnol. 8:297. doi: 10.3389/fbioe.2020.00297 Strandås, C., Kamal-Eldin, A., Andersson, R., and Åman, P. (2008). Phenolic glucosides in bread containing flaxseed. Food Chem. 110, 997–999. doi: 10.1016/j.foodchem.2008.02.088

Tanruean, K., and Rakariyatham, N. (2016). Efficient synthesis of 4-vinyl guaiacol via bioconversion of ferulic acid by Volvariella volvacea. Chiang Mai J. Sci. 43, 1212–1222.

Tressl, R., Renner, R., and Apetz, M. (1976). Volatile phenolic components in beer, smoked beer, and sherry. Zeitschrift Fur Lebensmittel-Untersuchung Und- Forschung 162, 115–122 doi: 10.1007/BF01274253

Vanbeneden, N., and Gils, F. (2008). Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. Food Chem. 107, 221–230. doi: 10.1016/j.foodchem.2007.08.008

Watanabe, S., Kanauchi, M., Takahashi, K., and Koizumi, T. (2009). Purification and characteristics of feruloyl decarboxylase produced by lactic acid bacteria from Awamori mash. J. Am. Soc. Brew. Chem. 65, 229–234 doi: 10.1094/ASBCJ-2009-0915-01

Yang, N., Huang, K., Lyu, C., and Wang, J. (2016). Pulsed electric field technology in the manufacturing processes of wine, beer, and rice wine: a review. Food Control. 61, 28–38 doi: 10.1016/j.foodcont.2015.09.022

Zeng, X. A., Yu, S. J., Zhang, L., and Chen, X. D. (2008). The effects of AC electric field on wine maturation. Innovat. Food Sci. Emerg. Technol. 9, 463–468. doi: 10.1016/j.ifset.2008.03.002

Zhang, B., Zeng, X. A., Sun, D., Yu, S. J., Yang, M. F., and Ma,

S. (2012). Effect of electric field treatments on brandy aging in Oak barrels. Food Bioprocess Technol. 6, 1635–1643. doi: 10.1007/s11947-012- 0788-7

Zhu, M., and Cui, Y. (2013). Determination of 4-vinylgaiacol and 4-vinylphenol in Top-fermented wheat beers by isocratic high performance liquid chromatography with ultraviolet detector. Braz. Arch. Biol. Technol. 56,1018–1023. doi: 10.1590/S1516-89132013000 600018

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Δημοσίευση III

Use of Pulsed Electric Field as a Low-Temperature and High-Performance "Green" Extraction Technique for the Recovery of High Added Value Compounds from Olive Leaves

Vasileios M. Pappas[1], Achillia Lakka[1], Dimitrios Palaiogiannis[1], Eleni Bozinou[1], George Ntourtoglou[1,2], Georgia Batra[1] , Vassilis Athanasiadis[1] , Dimitris P. Makris[1], Vassilis G. Dourtoglou[2] and Stavros I. Lalas[1]

1 **Department of Food Science & Nutrition, University of Thessaly, Terma N. Temponera Str.,43100 Karditsa, Greece; vpap@uth.gr (V.M.P.); achlakka@uth.gr (A.L.); dipaleog@med.uth.gr (D.P.); empozinou@uth.gr (E.B.); gntourtoglou@uniwa.gr (G.N.); gbatra@uth.gr (G.B.); vaathanasiadis@uth.gr (V.A.); dimitrismakris@uth.gr (D.P.M.)**

2 **Department of Wine, Vine & Beverage Sciences, School of Food Science, University of West Attica, Ag. Spyridonos Str., Egaleo, 12243 Athens, Greece; vdourt@uniwa.gr**

ABSTRACT

Olive leaves (OLL), an agricultural waste by-product, are considered a significant bioresource of polyphenols, known as bioactive compounds. This study evaluates the pulsed electric field (PEF) technique for the extraction of polyphenols from OLL. The study parameters included a series of "green" solvents (ethanol, water as well as mixtures of them at a 25% step gradient) and different input values for the pulse duration of PEF. The phytochemical extraction degree was evaluated using total phenol concentration (Folin–Ciocalteu method) and high-performance liquid chromatography (HPLC) analyses, while the antioxidant activity was assessed using differential scanning calorimetry (DSC). The results obtained from the PEF extracts were compared with those of the extracts produced without the PEF application. The highest PEF effect was observed for aqueous ethanol, 25% v/v, using a pulse duration of 10 μs. The increase in the total polyphenols reached 31.85%, while the increase in the specific metabolites reached 265.67%. The recovery in polyphenols was found to depend on the solvent, the pulse duration of treatment and the structure of the metabolites extracted.

Keywords: pulsed electric field; olive leaves; extraction optimization; polyphenols; green extraction

INTRODUCTION

Olive (*Olea europaea L*.) leaves (OLL) have a dual identity, both as waste material from the olive oil production or the olive tree-pruning season and as aromatic and medicinal herbs that are beneficial for human health [1,2]. It is well known that during the pruning of olives, either in December or in March, substantial leaf and branch masses are destroyed either by fire or fermentation or by composting. Based on available data from the literature [3–5], the global volume of wasted olive leaves reaches 12 Mt/year. This volume comes from pruning and olive oil production waste (~2 Mt/year), with most of it produced in Europe (~50%). Greece is third amongst European countries in yielding olive grove waste.

OLL contain a considerable amount of bioactive compounds belonging to the group known as polyphenols, such as phenolic acids, phenolic alcohols (hydroxytyrosol and tyrosol), flavonoids (luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, luteolin-4-O-glucoside), and secoiridoids (oleuropein) [5–8]. Among the aforementioned polyphenols, oleuropein is the most noted metabolite in OLL [7,9]. OLL have the highest level of oleuropein of all parts of the olive tree. Oleuropein is the most studied compound together with verbascoside, flavones, flavonols, flavan-3-ols, and other substituted phenols such as rutin. Most of the above compounds have been shown to possess hypo-cholesterolaemic properties [10]. In animal studies, dietary oleuropein from OLL resulted in hypo-cholesterolaemic activities [11]. Defensive action against the oxidation of low-density lipoproteins (LDL), implicated in the progress of atherosclerosis, as well as inhibition of the enzyme “3-hydroxy- 3- methyglutaryl coenzyme A”, a significant enzyme for cholesterol synthesis [12], are reported for hydroxytyrosol and oleuropein. The above effects of hydroxytyrosol and oleuropein are possibly related to the decreases in the levels of plasma cholesterol [13].

Numerous techniques have been developed to produce OLL extracts [14,15]. However, currently, there is no significant exploitation of these wastes, since the commonly used processes present serious disadvantages [16]. Conventional extraction is not considered suitable because it can lead to the thermal decomposition of various unstable metabolites. Additionally, it is non-selective, complicated, costly, and harmful for the environment (expensive and toxic organic solvents are demanded in large quantities, high temperatures and long extraction times are used, and the solvent must be evaporated) [17]. Different techniques, including supercritical fluid extraction, ultrasonically assisted solvent extraction, accelerated pressurized and microwave-assisted extraction, appear to also have serious disadvantages, such as

reduced recovery, high energy requirements and expensive equipment, making systematic, industrial production of bioactive compounds not feasible [18].

Pulsed electric field (PEF) is an emerging technique suitable for “green” sustainable production process development. The principle of PEF is to disintegrate the cell membrane structure for increasing extraction. During PEF, the phenomenon of electroporation (electrically induced formation of pores in the lipid bilayer under the influence of the induced transmembrane voltage) occurs periodically in a non-destructive manner for the cell under the effect of high-voltage pulsed electric field application. During PEF, the phenomenon of electroporation occurs periodically in a non-destructive manner for the cell. This way, an increased migration of intracellular water and solutes takes place to the external medium, resulting to enhanced mass transfer from the cell pores to the solution and therefore improvement of the recovery for the target compounds. Moreover, this non-thermal technology decreases processing time, reduces the degradation of unstable metabolites and energy costs, and, hence, improves the environmental impact [19]. The effectiveness of PEF treatment is directly related to the main processing factors such as electric field strength, pulse shape, duration, period, and specific energy.

PEF was initially applied for the non-thermal inactivation of microorganisms in liquid foods [20,21]. The utilization of the PEF technique for the recovery of bioactive phytochemicals was attempted first by Brodelius et al. [22] and is still limited. PEF has also been used before the extraction process to lower the operational costs, reduce the environmental impact, and achieve high yields of the desired compounds from a wide range of food processing wastes and by-products [9,23–29]. Another application of PEF was the extraction of flavor compounds from wood, resulting in the aging acceleration of young wine [30,31].

In our previous studies, we used pulsed electric field to extract polyphenols from Moringa oleifera leaves, aerial parts of Sideritis scardica, tepals of Crocus sativus, and fruits of Vitis vinifera [19,32], to increase bitter hops acids extraction rate [33], and to produce phenolic aromas in beer after enrichment of the beer wort with flax seeds [34].

In the present work, the PEF is proposed as a standalone extraction method of valuable bio-functional components, which can be applied in a simple “green” sustainable way. Moderate electric field intensity and relatively low energy input to succeed in the permeabilization of cell membranes by electroporation, preserving cell viability, were applied. Pure water, pure ethanol as well as their mixtures were used as extraction solvents, while PEF conditions that differentiated in pulse duration were tested to optimize the procedure. The use of PEF with solvent mixtures instead of pure water is a relatively new practice that can further increase the percentage of extraction. The purpose of the extraction solvent selection was to investigate the impact of the ratio of aqueous organic solvent on a potential PEF effect. Furthermore, the selectivity

of the abovementioned procedure (PEF + solvent composition) concerning the extraction of different compounds was evaluated. To the best of our knowledge, such a study has not been carried out previously. Finally, this study was performed keeping in mind that even though high concentrations of organic solvents, such as ethanol, lead to higher extraction yields, these solvents must be recovered and recycled, resulting in weighing down the final product with an enormous operating cost and the overall process with environmental implications. Therefore, an additional aim appeared: to substantially reduce the use of the organic solvent, ethanol, using PEF and, at the same time, allow the extraction of phenolic compounds in an economically feasible way and determine any possible selectivity concerning the extraction of different compounds.

MATERIALS AND METHODS

Chemicals

HPLC grade acetonitrile as well as formic acid (99%) were from Carlo Erba (Val de Reuil, France). Anhydrous sodium carbonate (99%) and gallic acid monohydrate were obtained from Penta (Prague, Czech Republic). Luteolin-7-O-glucoside, apigenin, rutin hydrate and oleuropein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (99.8%) and Folin–Ciocalteu reagent were purchased from Panreac (Barcelona, Spain).

Plant Material, Handling and Sample Preparation

The single variety of OLL used in this study was collected on the 12 October 2020 from a single 30-year-old olive tree (*Olea europaea* L., cv. Chondrolia Chalkidikis), in Karditsa Region, Greece (at 39◦21′46″ N and 21◦55′05″ E and elevation of 108 m, according to Google Earth version 9.124.0.1, Google, Inc., Mountain View, CA, USA). The plant material processing steps are illustrated in Figure 1. Before and after each extraction run, the temperature of the treatment chamber contents was measured. In all PEF-assisted extraction runs, the temperature increments due to the treatment never exceeded a ΔT of 1 ◦C.

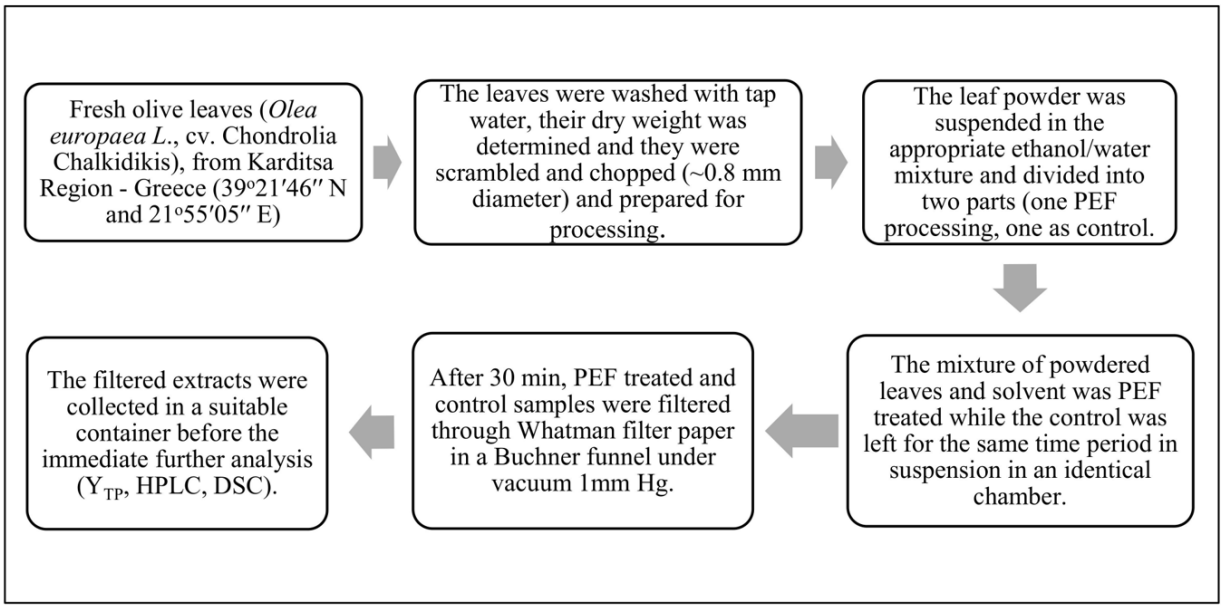


Figure 1. Plant material processing steps. Abbreviations: pulsed electric field (PEF); total phenol content (Y_{TP}); high performance liquid chromatography (HPLC); differential scanning calorimetry (DSC).

OLL Water Content Determination

For the determination of dry matter content, a sufficient quantity of pulverized leaves, was placed in a suitable container. After being weighted, the sample was dried in an oven (Binder BD56, Bohemia, NY, USA) at 105 ◦C for 6 h. The moisture content of the leaves was about 50%.

PEF System

The PEF system used (Figure 2) was a static bench-scale system, and its layout consisted of a high voltage power generator (Leybold, LD Didactic GmbH, Huerth, Germany) that could provide a maximum voltage of 25 kV, a 25 MHz Function/Arbitrary Waveform Generator (Siglent SDG1025, SIGLENT Technologies Germany GmbH, Augsburg, Germany), a tailored electronic switch circuit (series of Insulated gate bipolar transistors- IGBTs) and a rectangular custom-made stainless steel treatment chamber consisting of two identical flat parallel stainless-steel plates with dimensions of 10 cm x 10 cm separated at a uniform distance of 1 cm by a “Π” shaped Teflon

single piece that acted as an insulator. The effective volume of the treatment chamber was 80 mL.

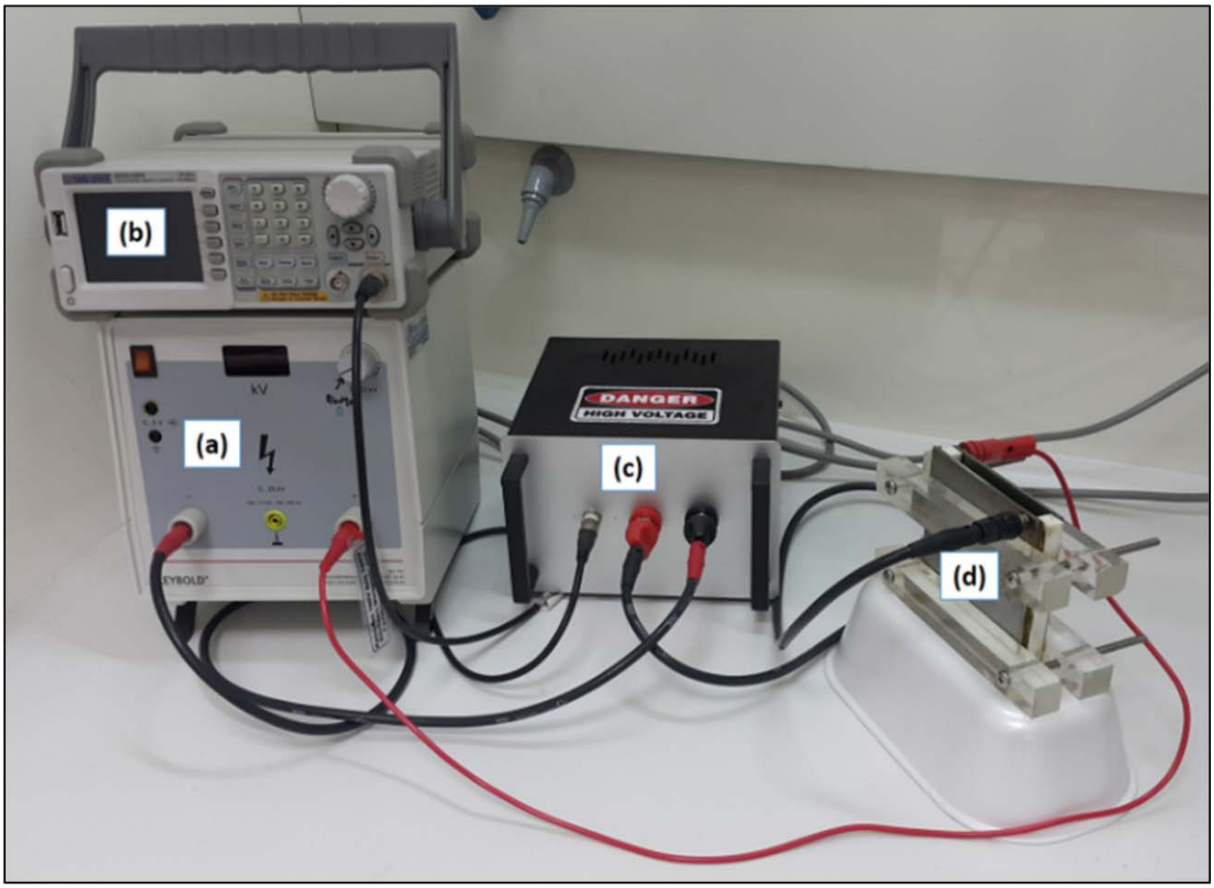


Figure 2. PEF System: (**a**) high-voltage power generator; (**b**) function/arbitrary waveform generator; (**c**) electronic switch circuit (IGBTs); (**d**) treatment chamber.

PEF Assisted Extraction Parameters and Calculus

In depth pre-screening of the main PEF parameters took place in order to investigate the optimal PEF parameters set for the specific system (plant material and solvent) and maximize polyphenolic content of the extracts. As main PEF parameters, we pre-examined the permeability controlling parameters, meaning field intensity (E), pulse duration (t_{Pulse}), and the pulse period (T) for a specified extraction time ($t_{Extraction}$). From the preliminary study (not reported here), our starting point (fixed variables) was pulse period (T): 1000 μs, electric field strength (E): 1 kV/cm, and extraction time ($t_{Extraction}$): 30 min in an aqueous ethanol solution. Based on the latter, our main study parameters included the extraction solvent and the PEF actual treatment time (variance of pulse

duration 10 μs and 100 μs) (please see also Table S1 in Supplementary Material). The series of the five green extraction solvents used was (i) water (0% EtOH), (ii) 25% v/v aqueous ethanol (25% EtOH), (iii) 50% v/v aqueous ethanol (50% EtOH), (iv) 75% v/v aqueous ethanol (75% EtOH) and v) ethanol (100% EtOH). The electrical conductivity of solvents was 2.3 μS/cm for 0% EtOH, 0.8 μS/cm for 25% EtOH, 0.3 μS/cm for 50% EtOH, 0.1 μS/cm for 75% EtOH and <0.1 μS/cm for 100% EtOH. Control extracts were prepared for comparison, following the same procedure as for PEF extracts, without the application of PEF. All extraction runs were carried out in triplicate.The ratio of the applied voltage, U, and the distance between the two electrodes, d (1 cm), was used to calculate the electric field strength, E = U/d, which was set at 1 kV/cm for all PEF assisted extraction runs. The pulse generator provided unipolar, rectangular- shaped pulses, with pulse duration (t_{Pulse}) varying between 10 and 100 μs under a period (T) of 1000 μs (Frequency = 1000 Hz), while the total extractiontime was 30 min, resulting in N = 1.8 x 10^6 total number of pulses, according to the design of this study. In each case of PEF-assisted extraction run, the actual PEF treatment time derived by the following equation:

$t = t_{Pulse} \times N$

where t_{Pulse} = pulse time duration (μs) and N = number of pulses (dimensionless).

Given that two different pulse times were investigated in the current study (t_{Pulse1} = 10 μs and t_{Pulse2} = 100 μs), the total PEF treatment time during the 30 min of each extraction was 18 and 180 s. The specific energy input Wspec (kJ/kg) for the PEF-assisted static extraction was derived from the following equation [35]:

$$W_{speec} = \sum_{0}^{N} \frac{N}{m} \int_{0}^{t_{Pulse}} U(t) \times I(t) dt$$

where N is the total number of pulses (dimensionless), m is the total weight of the sample (kg) poured into the PEF treatment chamber, t_{Pulse} is the duration of each pulse, U(t) is the output voltage, and I(t) the electric current applied to the sample.

For the application of PEF in a static homogenous solid–liquid extraction where the same pulse type and pulse time duration applied for each period, the values of U(t) and I(t) can be considered constant, simplifying the above function to the following equation for this study

$$W_{speec} = \frac{N}{m} \times U \times I \times t_{Pulse}$$

From the above equation, it was estimated that the energy input for all PEF assisted extractions was in the range of 0.155 and 1.55 kJ/kg, or 2.52 x10^{-6} and 2.52 x10^{-5} KWh, respectively.

Total Phenol Content of Extracts

The analysis was carried out using a validated protocol adopted by Lakka et al. [36].Total polyphenol yield (YTP) was calculated as follows:

$$Y_{TP}\left(mgGAE\,g^{-1}\,of\,dw\right) = \frac{C_{TP} \times V}{w}$$

where CTP is the total polyphenol concentration of the extract (mg L−1), V is the volume of the extraction medium (L) and w is the dry weight (g) of the plant material [37].

High-Performance Liquid Chromatography (HPLC)

A methodology previously described by Kaltsa et al. [6] was implemented. Chromatographic analyses were carried using a Shimadzu CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany), coupled to a Shimadzu SPD-M20A detector (Shimadzu Europa GmbH, Duisburg, Germany), and interfaced by Shimadzu LC solution software (Version 1.22 SP1) (accessed on 14th October 2020). The column used was a Phenomenex Luna C18(2) (100 Å, 5 µm, 4.6 × 250 mm) (Phenomenex, Inc., Torrance, CA, USA). Quantification was done with calibration curves (0–50 µg mL−1) constructed with rutin (R2 = 0.9990), luteolin-7-O-glucoside (R2 = 0.9980), apigenin (R2 = 0.9999) and oleuropein (R2 = 0.9990). Luteolin-7-O-glucoside and apigenin were quantified at 345 nm, rutin at 360 nm and oleuropein at 270 nm. The estimation of the total area the wavelength selected was 230 nm as it was considered the most representative for the compounds contained.

Differential Scanning Calorimetry (DSC)

Antioxidant activity was estimated using the DSC method according to Bobinaitė et al. [23], after the solvents' evaporation using a rotary evaporator. Determinations were conducted with a Perkin Elmer Diamond DSC (PerkinElmer Inc, Shelton, CT, USA). Oxygen was used as purge gas. In short, empty pans, hermetically sealed, were used as the control, while 4–5 mg of each sample was placed in DSC aluminum pans, closed with lids with a hole (1 mm in diameter), to allow the oxygen stream to be in contact with the sample. The temperature program was: hold for 1 min at 40 ◦C, heat from 40 to 200 ◦C (40 ◦C/min), and, finally, heat from 20 to 580 ◦C (20 ◦C/min). The

onset temperature of the oxidation peak considered as the starting temperature of oxidation (Tmax).

Statistical Analysis

Extractions as well as all determinations were performed three times. Statistical analysis was carried out using the Microsoft Excel 2019 (Redmond, WA, USA) software (Version 16.0.14026.20304) (accessed on 8th January 2021). Evaluation of the statistical significance (at $p < 0.05$) of the differences between mean values was also carried out.

RESULTS AND DISCUSSION

The extraction of valuable compounds from plant tissues and the evaluation of different techniques to manage this challenge were extensively studied. Yasemi et al. [38] reported Soxhlet extraction to show the highest recovery (62%) among different conventional techniques, such as maceration, ultrasound and microchannels. The optimum solvent for the Soxhlet technique, among different combinations tested, was ethanol:water (80:20). However, even higher recoveries, from 80 to 95%, were reached by emerging techniques that implicate supercritical fluid extraction combined with pressurized liquid extraction and high temperatures combined with pressurized liquid extraction [39,40]. According to the study of Delsart et al. [41], an enhanced extraction in anthocyanins (19%) of Cabernet Sauvignon grapes before fermentation was achieved via the PEF treatment. Fincan et al. [42] applied PEF treatment to extract red pigment from red beetroot tissue and reached a release of total red coloring and ionic content of about 90%.

Total Phenol Content of the Extracts-Solvent/Water Composition Extraction Evaluation

Initially, a screening of the optimal ethanol/water ratio was carried out by choosing the highest difference in total phenol content between the samples treated with PEF and control. The results are presented in Figures 3 and 4. As expected, the highest extraction rate was achieved by PEF and Control samples with 50% and 75% EtOH (no significant difference shown between them). However, the highest percentage increase in YTP between PEF and the control sample was achieved by means of the addition of 25% EtOH. A significant ($p < 0.05$) increase in YTP for PEF extract (compared to the control extract) was observed following the addition of 25% EtOH, which reached the maximum of 31.85%. YTP for the 25% EtOH PEF extract was

20.75 mg GAE g−1 dw, while for the 25% EtOH control extract, it was 15.74 mg GAE g−1 dw.

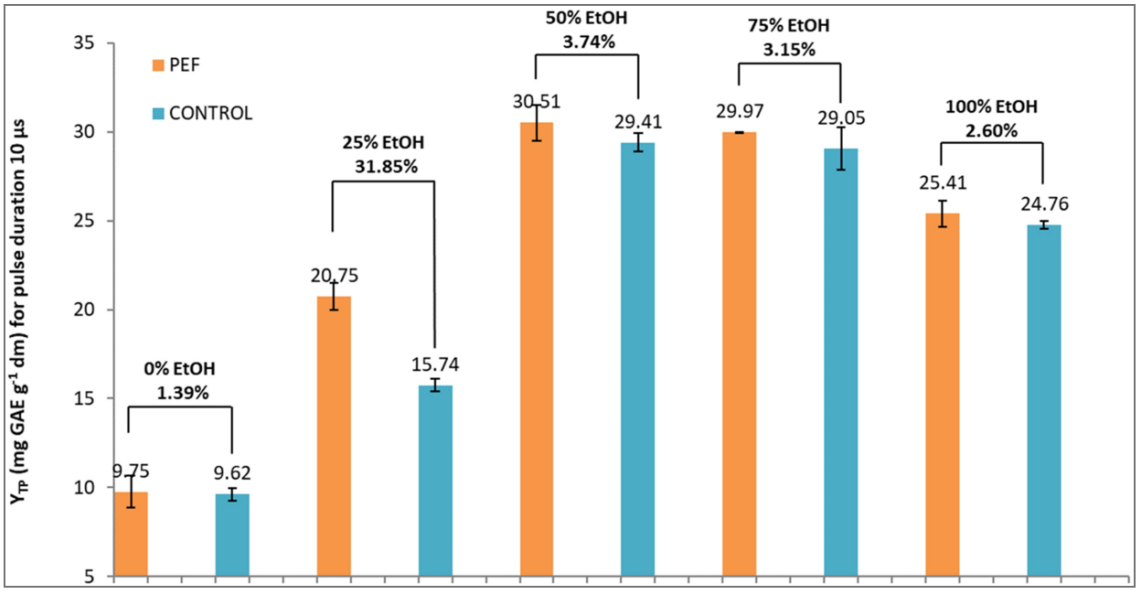


Figure 3. Y_{TP} (mg of gallic acid equivalents (GAE) per gram of dry weight (dw)) for PEF and control samples in five different tested solvents and a pulse duration of 10 µs.

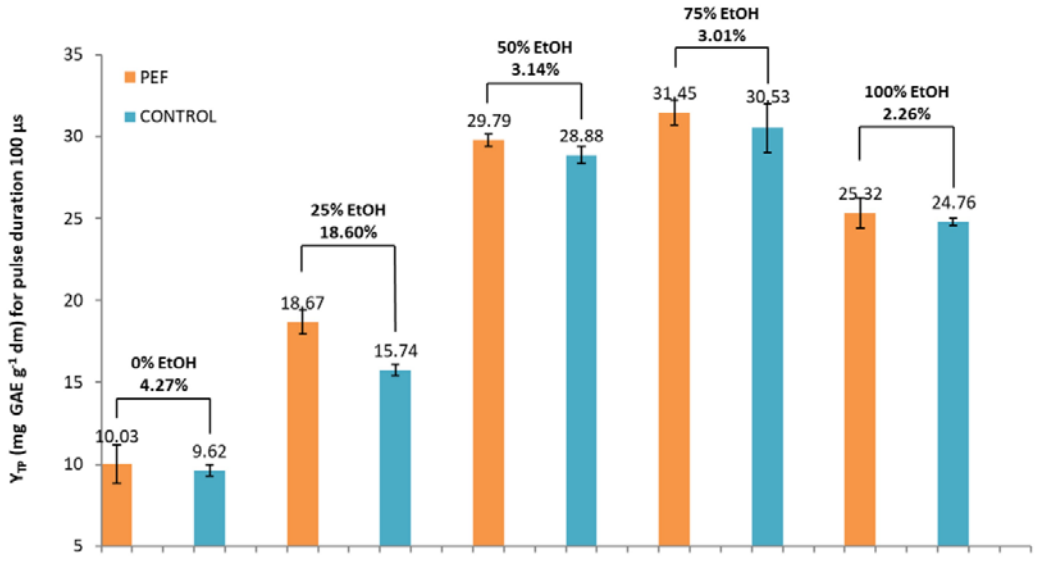


Figure 4. Y_{TP} (mg of gallic acid equivalents (GAE) per gram of dry weight (dw)) for PEF and control samples in five different tested solvents and a pulse duration of 100 µs.

Next, a second set of PEF conditions was used. The pulse duration was increased from 10 to 100 µs, while the other PEF parameters, as well as induction time, were kept

constant. The extracts were prepared using the same solvents. The results regarding the effect of EtOH addition for a pulse duration of 100 μs were similar as for a pulse duration of 10 μs. Again, the highest extraction efficiency was achieved by PEF and control samples with 50% and 75% EtOH. At a pulse duration of 100 μs, the solvent 25% EtOH, resulted again in the higher percentage increase (significant at $p < 0.05$) in YTP between PEF and control sample. YTP at this EtOH content (25%) was lower (by 10.02%) than the corresponding yield reached with a PEF condition of 10 μs. According to the results, the highest difference is ultimately in the samples treated with 25% ethanol. It seems that any percentage losses in yield, caused by the solvent choice, were balanced from the application of the PEF. Another point of interest is that the application of a large number of short-duration pulses seems to lead to higher efficiencies of total phenolic content recovery.

Differential Scanning Calorimetry (DSC)

DSC is a thermal analysis technique used to assess the changes in the different physic-ochemical properties of materials and their relationship with the heat flow delivered by the instrument [43]. As reported by the same authors, the DSC method can be used to determine the oxidative stability of antioxidants. Relying on the measurements of the incubation period, oxidative stability can be estimated by the extrapolated temperature at the beginning of the oxidation process. Therefore, DSC can be used to determine the kinetic parameters of an oxidation from the resulting thermographic curves which reveal the temperature of the extrapolated onset of the thermo-oxidation process [43]. Specifically, Tmax is considered the highest oxidation peak of the thermographic curve. The higher the Tmax appears to be, the higher the resistance of the sample is.

During this study, the exothermic peaks of the extracts were measured in the range of 40 to 580 ◦C, relating to their autoxidation process. According to the results (Table 1), the maximum peak oxidation (Tmax) was 569 ◦C, which was achieved by the samples treated by pulse duration of 100 μs, a pulse period of 1000 μs, an electric field strength of 1 kV/cm, and an induction time of 30 min with addition of 75% EtOH. These samples

presented the higher oxidation resistance and, therefore, the higher antioxidant activity. All the results of the DSC determinations showed that the Tmax was directly related to the total phenol content of each sample, while, concerning the difference in extraction efficiency, the highest (significant at $p < 0.05$) was presented between control samples and PEF samples treated by a pulse duration of 10 μs with 25% EtOH (15.16%), followed by the PEF samples treated with a pulse duration of 100 μs (8.56%) and 75% EtOH (both pulse durations). No significant differences were observed when 0% and 100% EtOH (both pulse durations) and 50% EtOH (100 μs) were used.

Table 1. DSC results on temperature (˚C) of oxidation (T_{max}) of the various samples.

Extraction Solvent Synthesis	PEF Pulse Duration	PEF Treated		Control		Increase (%)
		Average	SD	Average	SD	
0% EtOH	10 μs	238	2	233	4	2.15
	100 μs	241	5			3.43
25% EtOH	10 μs	471	6	409	3	15.16
	100 μs	444	2			8.56
50% EtOH	10 μs	565	5	549	6	2.91
	100 μs	557	5			1.46
75% EtOH	10 μs	560	4	540	6	3.70
	100 μs	569	5			5.37
100% EtOH	10 μs	518	3	512	3	1.17
	100 μs	516	2			0.78

In Figures 5–7, the thermograms of control samples (no PEF applied) and PEF-treated samples in five different tested solvents are displayed. It appears that all the curves of the thermograms have different shapes. This possibly denotes a different composition of each extract, meaning that each and every extract is unique and contains a different proportion of active compounds or, maybe, a different composition of compounds. This is a result that should have been expected due to the different conditions used for the extraction.

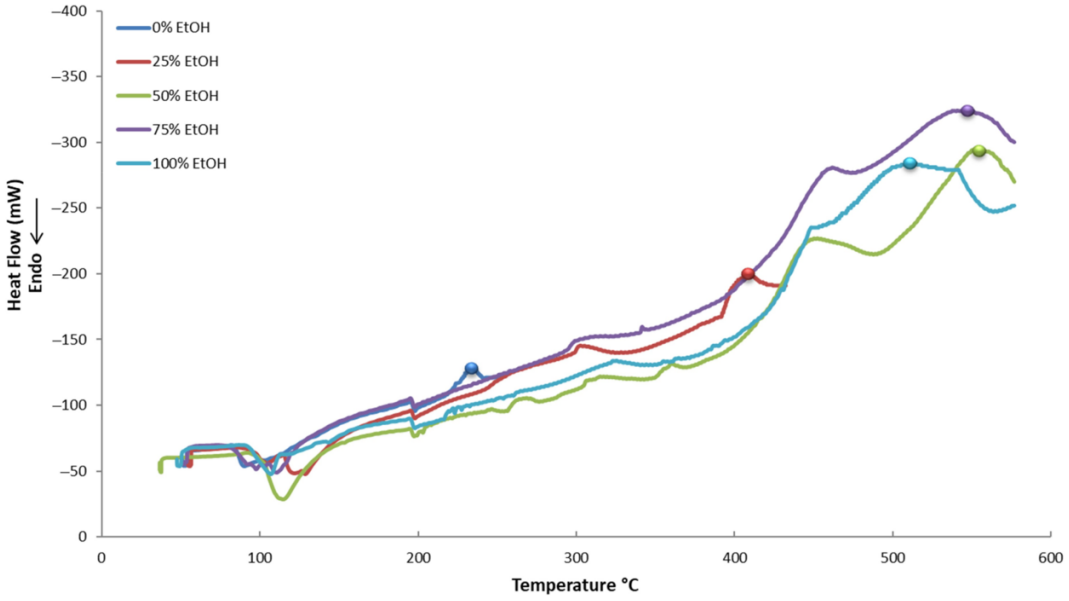


Figure 5. Differential scanning calorimetry (DSC) thermograms of control samples (no PEF applied) in five different tested solvents.

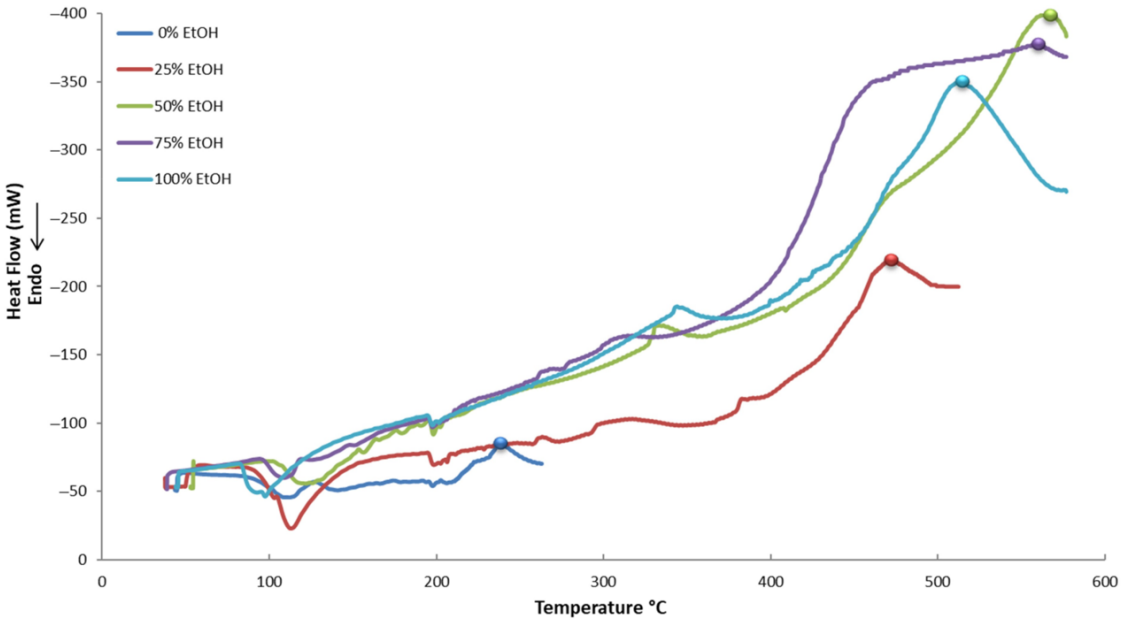


Figure 6. Differential scanning calorimetry (DSC) thermograms of PEF-treated (10 μs) samples in five different tested solvents.

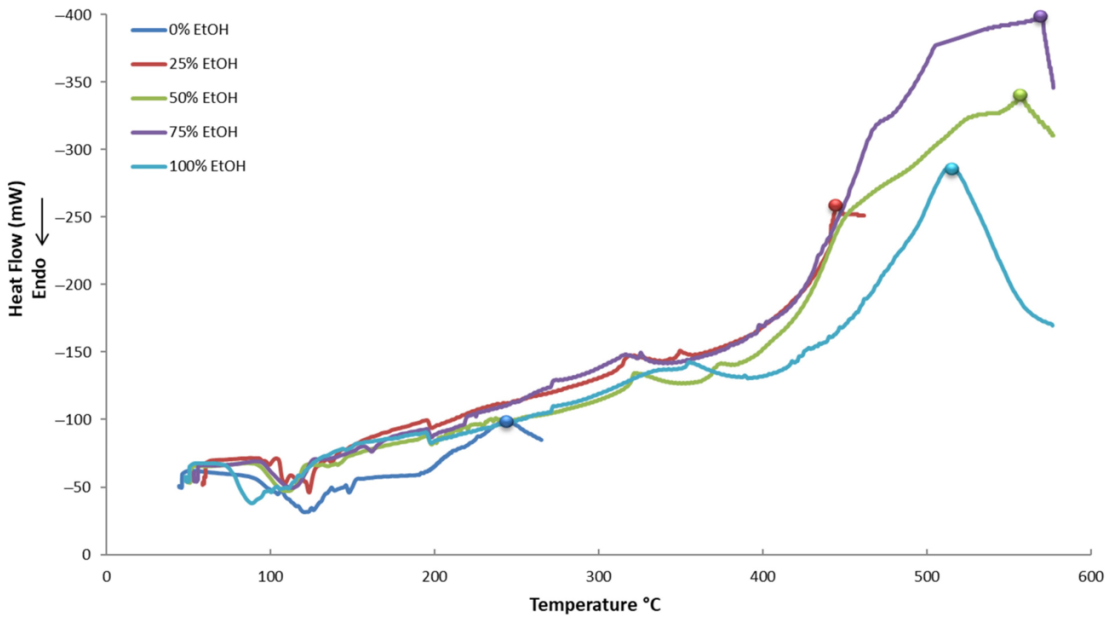


Figure 7. Differential scanning calorimetry (DSC) thermograms of PEF-treated (100 μs) samples in five different tested solvents.

Characterization of the Extracts Using HPLC

Estimation of PEF Effect by Determination of Extracts Total Area

The results based on the estimated total area are in accordance with the phenol content determination results. The PEF condition with a pulse duration of 10 μs in 25% EtOH led to the highest percentage difference for the total area between PEF and the control sample (please see also Figures S1 and S2 in Supplementary Material). Specifically, the total area for the optimum condition of a pulse duration of 10 μs in 25% EtOH was 44,156,899 for the PEF sample and 36,566,097 for the control sample, reflecting a significant ($p < 0.05$) percentage increase of 20.76%. The corresponding percentage increase for the PEF condition with a pulse duration of 100 μs was 18.38% (again significant at $p < 0.05$). For pure water, the PEF condition with a pulse duration of 10 μs was also significantly ($p < 0.05$) superior to the PEF condition with a pulse duration of 100 μs. Percentage increases over PEF sample for pure water reached 17.44% and 7.38%, respectively, for the two PEF conditions. A gradual increase in EtOH content up to 100% did not result in a corresponding percentage increase in the total area over the PEF sample. On the contrary, the addition of EtOH to water in percentages higher than 25% resulted in a gradual reduction (however, not significant at $p < 0.05$) in the percentage increase in the total area over the PEF sample. When extracting with pure EtOH, the PEF effect for both conditions of pulse duration is minimized and considered not significant.

Polyphenolic Composition

According to the literature, luteolin and its related compounds are the most frequently found flavonoids in olive leaf water and EtOH extracts, while the most abundant phytochemicals are luteoline-7-O-glucoside and oleuropein. Quercetin-3-O-rutinoside and apigenin-7-O-rutinoside have also been identified [5–8,44,45]. The results of the current study are in accordance with the above relevant literature. The chromatograms (Figure 8) revealed the existence of six principal constituents. Peaks 2 (λmax at 351 nm) and 4 (λmax at 347 nm) were identified as quercetin-3-O-rutinoside and luteolin-7-O-glucoside, respectively, comparing their retention time and absorption spectrum to those of the corresponding reference substances. Peaks 5 and 6 were tentatively identified in our previous work [6] as apigenin-7-O-rutinoside and luteolin-3′-O-glucoside, respectively. Peaks 1, 3 and 7 could not be identified due to a lack of corresponding reference substances. However, according to their typical flavone UV-VIS spectrum (please see also Figure S3 in Supplementary Material) and bibliographic data [5,6], they are believed to be luteolin-related compounds (luteolin diglucoside, luteolin rutinoside and luteolin aglycone, respectively). Peak 8 with a λmax at 280 nm was identified as oleuropein following the comparison of its retention time and absorption spectrum with the corresponding values of the reference substance. Luteoline-7-O-glucoside and oleuropein indeed appear to be the predominant phytochemicals. Their content in the control samples extracts prepared in 25% EtOH amounted to 0.48 and 0.58 mg g−1 dw, respectively. Quercetin-3-O-rutinoside, apigenin-7-O-rutinoside and luteolin-3′-O-glucoside had lower contents of 0.07, 0.14 and 0.2 mg g−1 dw, respectively (please see also Table S2 in Supplementary Material).

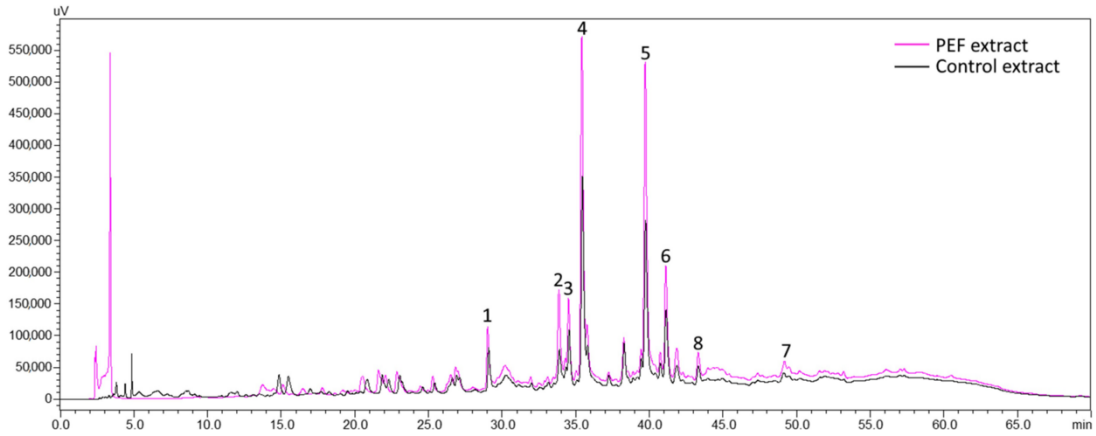


Figure 8. Overlay of chromatograms of PEF and control extracts with a pulse duration of 10 μs and extraction solvent 25% EtOH. Peak 1: not identified, Peak 2: quercetin-3-O-rutinoside, Peak 3: not identified, Peak 4: luteolin-7-O-glucoside, Peak 5: apigenin-7-O-rutinoside, Peak 6: luteolin-3′-O-glucoside, Peak 7: not identified, Peak 8: oleuropein.

Comparing the extraction efficiency between the set of PEF conditions utilizing pulses of 10 and 100 μs, the 10 μs pulses showed better performance in the extraction of phenolics. It is obvious that PEF treatment has a remarkable positive effect in the

recovery of these compounds, reaching an increase of up to 265.67%. The above observations are illustrated comparatively in Figures 9 and 10 (in 25% EtOH), and confirm an adequately reported observation of a short-duration pulse disintegration effect on the membranes and intracellular compounds extraction rate from vegetable cells. Looking at the above figures, it can be concluded that the pulse time affects the extraction and probably has to do with the structure of the molecules being extracted. For compounds such as phenolic glycosides, where the molecule usually comprises one or two sugar units bound to a flavone-backbone (quercetin), the time of 10 μs gave significantly ($p < 0.05$) better results for all compounds apart from oleuropein (no significant difference). Concerning oleuropein, the longer time of 100 μs gave significantly ($p < 0.05$) better results. Looking at the molecular structures, oleuropein is different from other phenolic compounds since it has no flavone backbone and is smaller in size. Various compounds' solubility is a key factor in samples treated with PEF. Regarding solubility, quercetin-3-O-rutinoside has a low water solubility of 0.125 g L−1, luteolin-7- O-glucoside of 1.08 g L−1, apigenin of 0.97 g L−1, and oleuropein of 0.73 g L−1. Ethanol is an excellent solvent for oleuropein (solubility: 30 g L−1), but all other glycosides are less soluble in it (i.e., rutin has a solubility of 5.5 g L−1 at room temperature). Therefore, the choice of different PEF conditions (pulses of 10 or 100 μs) can facilitate the selective extraction of different molecules from plant material. This is highly important since the selective extraction of compounds is often a tedious, time- and energy-consuming procedure. According to Figure 11, where a comparison of the effect (% increase) of the pulse time duration on the extraction of various compounds is presented, oleuropein is the only molecule whose extraction appears higher under a 100 μs pulse. This result leads us to the conclusion that the 10 μs pulses in a period of 1000 Hertz affect the less soluble molecules (i.e., rutin) to a greater extent. The larger molecules possibly need more pulses to be extracted and, therefore, a combination of molecular size and solubility has to be considered for their selective extraction. The thermographic curves during DSC analysis (Figures 5–7) also show differences in the resistance to oxidation of the extracts, possibly due to their composition in active compounds. This is caused by the extracting ability of each extraction procedure.

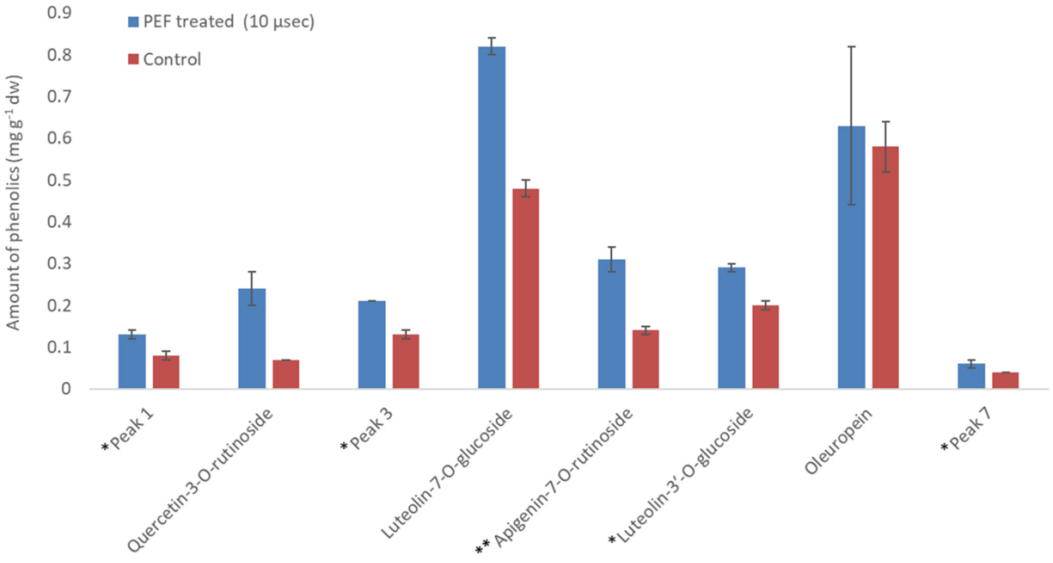


Figure 9. Amount of phenolics (mg g^{-1} dw) in PEF-treated leaves versus control at 10 μs pulse duration and 25% EtOH. * Luteolin-3´-O-glucoside and peaks 1,3 and 7 were quantified as luteolin-7-O-glucoside; ** Apigenin-7-O-rutinoside was quantified as apigenin.

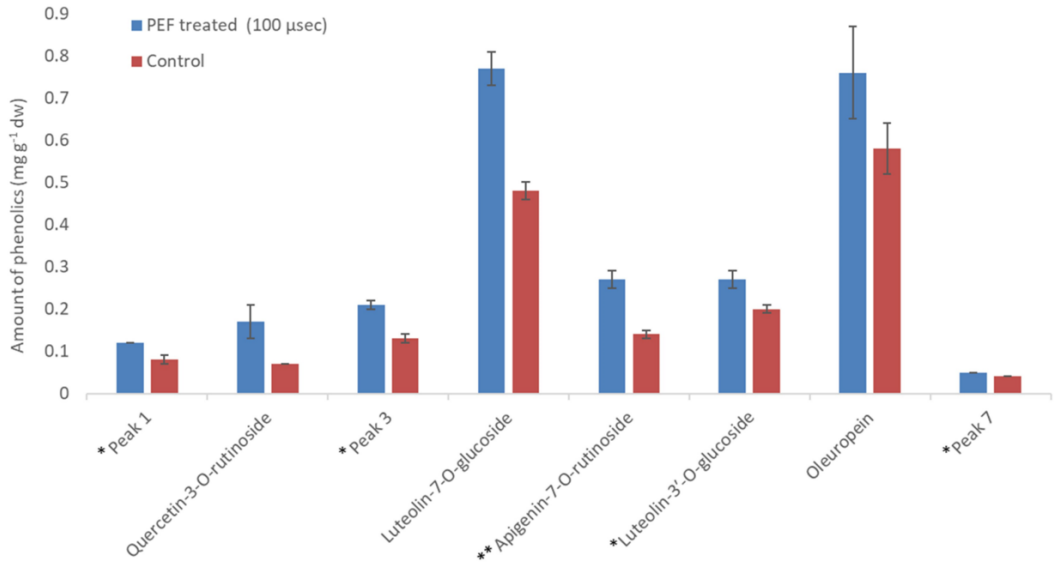


Figure 10. Amount of phenolics (mg g^{-1} dw) in PEF treated leaves versus control at 100 μs pulse duration and 25% EtOH. * Luteolin-3´-O-glucoside and peaks 1, 3 and 7 were quantified as luteolin-7-O-glucoside; ** Apigenin-7-O-rutinoside was quantified as apigenin.

The significance of the results presented above are further illustrated based on the fact that in the current study, the 71.87% increase, for the PEF condition with pulse duration 10 μs, in the case of the basic metabolite luteolin-7-O-glucoside, led to an amount of

0.82 mg g^{-1} dw in the extract. The exact same amount was reached by Palmeri et al. [45]

using water at 80 °C as the extraction solvent with a liquid to solid ratio of 20:1 mL g^{-1}. This result is considered significant as, in the current study, we conducted a non-thermal extraction, using a much lower liquid to solid ratio (just 2.5:1 *w*/*w*), with the addition of a green organic solvent (EtOH) in a percentage of only 25%. Regarding the secondary metabolites, quercetin-3-O-rutinoside, apigenin-7-O-rutinoside, and luteolin-3′-O-glucoside, we also succeeded in extracting amounts around 0.3 mg g^{-1} dw. This quantity has also been reported in previous studies [6,45] using energy demanding extraction methods and a significant quantity of extraction solvent.

PEF processing factors, such as electric field strength, pulse shape, duration, period, and specific energy, could be further optimized in a future work to maximize polyphenol concentration and explore the possibility of selective extraction. Another point of interest for future work is to further investigate the role of solvent polarity to the PEF effect.

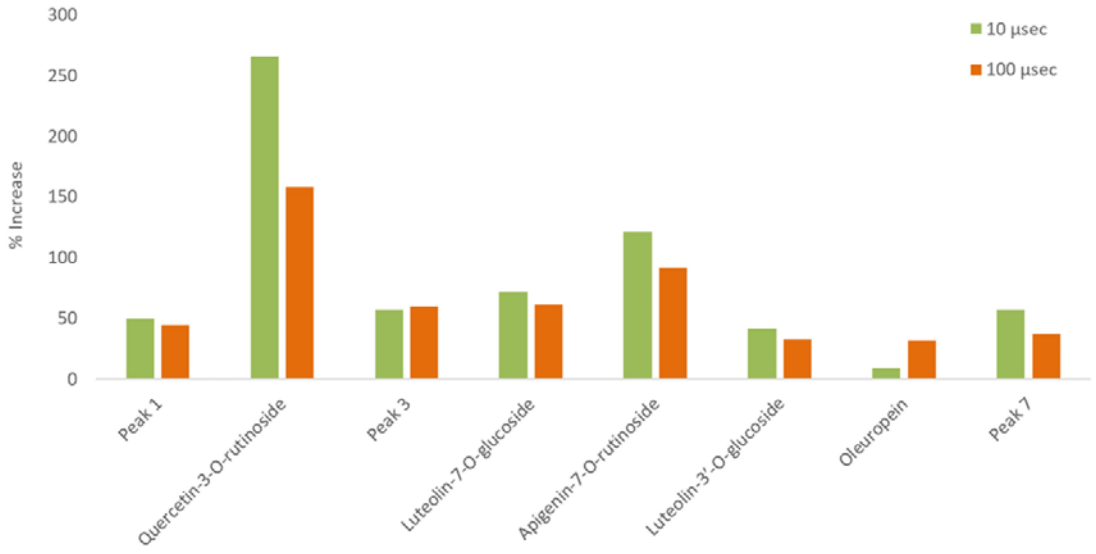


Figure 11. Comparison of the effect (% increase) of different pulse time durations on the extraction of various compounds in the same ethanol/water ratio (25% EtOH).

CONCLUSIONS

This study aimed to extract polyphenols from olive leaves using the PEF technique and to suggest a method that would substantially reduce the use of the organic solvent ethanol (used in a conventional extraction techniques), replacing it with a technology that would allow us to isolate phenolic compounds in an economically feasible way. During the experiments, different solvent mixtures and different PEF

conditions were applied. The novelty of the research includes the selection of an aqueous organic solvent with the lowest possible ratio of ethanol and the development of the PEF technique as a standalone static solid liquid extraction process. Although higher green solvent ratios gave an increased extraction rate, a PEF-assisted procedure with a much lower ratio of EtOH (25%) showed the maximum increase in the total yield in polyphenols. Therefore, one of the aims of this work, the substantial reduction in the quantity of solvent, was fulfilled. According to the results, olive leaves' PEF extracts possess higher content in total polyphenols. PEF application (pulse duration: 10 μs, pulse period: 1000 μs, electric field strength: 1 kV/cm, and induction time: 30 min) in 25% EtOH, increased the total yield in polyphenols up to 31.85%. The recovery of major and secondary metabolites was enhanced by PEF treatment up to 265.67%. The amount of the extracted polyphenols was a function of the solvent, the pulse duration of treatment, as well as the structure of the metabolites extracted and their solubility. The DSC results show that the T_{max} was directly related to the total phenol content of each sample. In conclusion, PEF treatment increased the recovery of polyphenols. This indicates that PEF presents excellent potential for "green" selective extraction of biofunctional constituents from olive leaves (phenolic compounds) that can be of further use in functional food manufacturing in a sustainable way, producing high quality products that present numerous public health benefits.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/beverages7030045/s1, Figure S1: HPLC total area for PEF and Control samples in five different tested solvents and a pulse duration of 10 μsec, Figure S2: HPLC total area for PEF and Control samples in five different tested solvents and a pulse duration of 100 μsec. Figure S3: UV-Vis spectras obtained by HPLC-DAD analysis of a) peak 1, b) peak 3, c) peak 7 and d) Luteolin-7-O-glucosidereference substance. Table S1: Extraction conditions and PEF procedure parameters. Table S2: Averages (mg g^{-1} dw) of major compounds of olive leaf PEF treated and control extracts, prepared with 25% ethanol.

Author Contributions: Conceptualization, S.I.L. and V.G.D.; Methodology, V.M.P., G.N. and A.L.; Validation, A.L.; Formal Analysis, A.L., D.P., E.B., G.B. and V.A.; Investigation, V.M.P., A.L., D.P., E.B., G.B., G.N. and V.A.; Resources, S.I.L.; Data Curation, V.M.P., A.L. and E.B.; Writing—Original Draft Preparation, A.L., V.M.P. and D.P.; Writing—Review and Editing, S.I.L., V.G.D., V.A. and E.B.; Supervision, S.I.L., V.G.D. and D.P.M.; Project Administration, S.I.L.; Funding Acquisition, S.I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and

Innovation, under the call RESEARCH- CREATE-INNOVATE (project code: T1EDK-03762).

Data Availability Statement: The data presented in this study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Sahin, S.; Bilgin, M. Olive tree (Olea europaea L.) leaf as a waste by-product of table olive and olive oil industry: A review. J. Sci. Food Agric. 2018, 98, 1271–1279. [CrossRef] [PubMed]

Toledano, A.; Alegria, I.; Labidi, J. Biorefining of olive tree (Olea europea) pruning. Biomass Bioenerg. 2013, 59, 503–511. [CrossRef]

Khdair, A.; Abu-Rumman, G. Sustainable environmental management and valorization options for olive mill byproducts in the Middle East and North Africa (MENA) region. Processes 2020, 8, 671. [CrossRef]

Romero, I.; Ruiz, E.; Castro, E.; Moya, M. Acid hydrolysis of olive tree biomass. Chem. Eng. Res. Des. 2010, 88, 633–664. [CrossRef]

Herrero, M.; Temirzoda, T.N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. J. Chromatogr. A 2011, 1218, 7511–7520. [CrossRef]

Kaltsa, O.; Grigorakis, S.; Lakka, A.; Bozinou, E.; Lalas, S.; Makris, D.P. Green valorization of olive leaves to produce polyphenol- enriched extracts using an environmentally benign deep eutectic solvent. AgriEngineering 2020, 2, 226–239. [CrossRef]

Lalas, S.; Athanasiadis, V.; Gortzi, O.; Bounitsi, M.; Giovanoudis, I.; Tsaknis, J.; Bogiatzis, F. Enrichment of table olives with polyphenols extracted from olive leaves. Food Chem. 2011, 127, 1521–1525. [CrossRef]

Mylonaki, S.; Kiassos, E.; Makris, D.P.; Kefalas, P. Optimization of the extraction of olive (Olea europaea) leaf phenolics using water/ethanol-based solvent systems and RSM. Anal. Bioanal. Chem. 2008, 392, 977–985. [CrossRef] [PubMed]

Briante, R.; La Cara, F.; Febbraio, F.; Patumi, M.; Nucci, R. Bioactive derivatives from oleuropein by a biotransformation on Olea europaea leaf extracts. J. Biotechnol. 2002, 93, 109–119. [CrossRef]

Romani, A.; Mulinacci, N.; Pinelli, P.; Vincieri, F.F.; Cimato, A. Polyphenolic content in five tuscany cultivars of Olea europaea L. J. Agric. Food Chem. 1999, 47, 3. [CrossRef] [PubMed]

Sarica, S.; Toptas, S. Effects of dietary oleuropein supplementation on growth performance, serum lipid concentrations, and lipid oxidation of Japanese quails. J. Anim. Physiol. Anim. Nutr. 2014, 98, 1176–1186. [CrossRef]

Cayan, H.; Erener, G. Effect of olive leaf (Olea europaea) powder on laying hens performance, egg quality, and egg yolk cholesterol levels. Asian Australas. J. Anim. Sci. 2015, 28, 538–543.

Patrick, L.; Uzick, M. Cardiovascular disease: C-reactive protein and the inflammatory disease paradigm: HMG-CoA reductase inhibitors, alpha-tocopherol, red yeast rice, and olive oil polyphenols. A review of the literature. Altern. Med. Rev. 2001, 6, 248–271. [PubMed]

Otero, D.M.; Oliveira, F.M.; Lorini, A.; Antunes, B.F.; Oliveira, R.M.; Zambiazi, R.C. Oleuropein: Methods for extraction, purifying and applying. Rev. Ceres 2020, 67, 315–329. [CrossRef]

Mahugo Santana, C.; Sosa Ferrera, Z.; Esther Torres Padrón, M.; Juan Santana Rodríguez, J. Methodologies for the extraction of phenolic compounds from environmental samples: New approaches. Molecules 2009, 14, 298. [CrossRef] [PubMed]

Araújo, M.; Pimentel, F.B.; Alves, R.C.; Oliveira, M.B.P.P. Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. Trends Food Sci. Tech. 2015, 45, 200–211. [CrossRef]

Lamprou, G.K.; Vlysidis, A.; Tzathas, K.; Vlyssides, A.G. Statistical optimization and kinetic analysis of the extraction of phenolic compounds from olive leaves. J. Chem. Technol. Biotechnol. 2020, 95, 457–465. [CrossRef]

Ferrera, Z.S.; Sanz, C.P.; Santana, C.M.; Rodríguez, J.J.S. The use of micellar systems in the extraction and pre-concentration of organic pollutants in environmental samples. TrAC Trend. Anal. Chem. 2004, 23, 469–479. [CrossRef]

Bozinou, E.; Karageorgou, I.; Batra, G.; Dourtoglou, V.; Lalas, S. Pulsed electric field extraction and antioxidant activity determination of Moringa oleifera dry leaves: A comparative study with other extraction techniques. Beverages 2019, 5, 8. [CrossRef]

Álvarez, I.; Pagán, R.; Condon, S.; Raso, J. The influence of process parameters for the inactivation of Listeria monocytogenes by pulsed electric fields. Int. J. Food Microbiol. 2003, 87, 87–95. [CrossRef]

Grahl, T.; Märkl, H. Killing of microorganisms by pulsed electric fields. Appl. Microbiol. Biotechnol. 1996, 45, 148–157. [CrossRef]

Brodelius, P.E.; Funk, C.; Shillito, R.D. Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored secondary products. Plant. Cell Rep. 1988, 7, 186–188. [CrossRef] [PubMed]

Bobinaite˙, R.; Pataro, G.; Lamanauskas, N.; Šatkauskas, S.; Viškelis, P.; Ferrari, G. Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products. J. Food Sci. Tech. 2015, 52, 5898–5905. [CrossRef] [PubMed]

Carullo, D.; Pataro, G.; Ferrari, G. Effect of PEF pre-treatment and extraction temperature on the recovery of carotenoids from tomato wastes. Chem. Eng. Trans. 2019, 75, 139–144.

Hendrawan, Y.; Larasati, R.; Wibisono, Y.; Umam, C.; Sutan, S.; Hawa, L. Extraction of phenol and antioxidant compounds from kepok banana skin with PEF pre-treatment. IOP Conf. Ser. Earth Environ. Sci. 2019, 305, 012065. [CrossRef]

Luengo, E.; Álvarez, I.; Raso, J. Improving carotenoid extraction from tomato waste by pulsed electric fields. Front. Nutr. 2014, 1,

12. [CrossRef]

Martín- García, B.; Tylewicz, U.; Verardo, V.; Pasini, F.; Caravaca, A.M.G.; Caboni, M.; Rosa, M.D. Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers' spent grains. Innov. Food Sci. Emerg. 2020, 64, 102402. [CrossRef]

Pataro, G.; Carullo, D.; Siddique, A.; Falcone, M.; Donsì, F.; Ferrari, G. Improved extractability of carotenoids from tomato peels as side benefits of PEF treatment of tomato fruit for more energy-efficient steam-assisted peeling. J. Food Eng. 2018, 233. [CrossRef]

Sukardi, S.; Purwaningsih, I.; Sita, P. Extraction of phenolic compounds from basil (Ocimum americanum L.) leaves with pretreatment using pulsed electric field (PEF). IOP Conf. Ser. Earth Environ. Sci. 2020, 475, 012056. [CrossRef]

Drosou, F.; Yang, E.; Marinea, M.; Dourtoglou, T.; Chatzilazarou, A.; Dourtoglou, V. An assessment of potential applications with pulsed electric field in wines. In BIO Web of Conferences; EDP Sciences: Les Ulis Cedex, France, 2017; Volume 9. [CrossRef]

Zeng, X.A.; Yu, S.; Zhang, L.; Chen, X. The effects of AC electric field on wine maturation. Innov. Food Sci. Emerg. 2008, 9, 463–468.

[CrossRef]

Lakka, A.; Bozinou, E.; Makris, D.P.; Lalas, S.I. Evaluation of pulsed electric field polyphenol extraction from Vitis vinifera, Sideritis scardica and Crocus sativus. Chemengineering 2021, 5, 25. [CrossRef]

Ntourtoglou, G.; Tsapou, E.A.; Drosou, F.; Bozinou, E.; Lalas, S.; Tataridis, P.; Dourtoglou, V. Pulsed electric field extraction of α and β-acids from pellets of Humulus lupulus (hop). Front. Bioeng. Biotechnol. 2020, 8, 297. [CrossRef] [PubMed]

Tsapou, E.A.; Ntourtoglou, G.; Drosou, F.; Tataridis, P.; Dourtoglou, T.; Lalas, S.; Dourtoglou, V. In situ creation of the natural phenolic aromas of beer: A pulsed electric field applied to wort-enriched flax seeds. Front. Bioeng. Biotechnol. 2020, 8, 1219. [CrossRef]

Roselló-Soto, E.; Barba, F.; Parniakov, O.; Galanakis, C.; Lebovka, N.; Grimi, N.; Vorobiev, E. High voltage electrical discharges, pulsed electric field, and ultrasound assisted extraction of protein and phenolic compounds from olive kernel. Food Bioprocess Tech. 2014, 8, 885–894. [CrossRef]

Lakka, A.; Grigorakis, S.; Karageorgou, I.; Batra, G.; Kaltsa, O.; Bozinou, E.; Lalas, S.; Makris, D.P. Saffron processing wastes as a bioresource of high-value added compounds: Development of a green extraction process for polyphenol recovery using a natural deep eutectic solvent. Antioxidants 2019, 8, 586. [CrossRef]

Karakashov, B.; Grigorakis, S.; Loupassaki, S.; Makris, D.P. Optimisation of polyphenol extraction from Hypericum perforatum (St.

John's Wort) using aqueous glycerol and response surface methodology. J. Appl. Res. Med. Aromat. Plants 2015, 2, 1–8. [CrossRef]

Yasemi, M.; Heydarinasab, A.; Rahimi, M.; Ardjmand, M. Microchannels effective method for the extraction of oleuropein compared with conventional methods. J. Chem. 2017, 2017, 6594156. [CrossRef]

Xynos, N.; Papaefstathiou, G.; Psychis, M.; Argyropoulou, A.; Aligiannis, N.; Skaltsounis, L. Development of a green extraction procedure with super subcritical fluids to procedure extracts enriched in oleuropein from olive leaves. J. Supercrit. Fluid 2012, 67, 89–93. [CrossRef]

Xynos, N.; Papaefstathiou, G.; Gikas, E.; Argyropoulou, A.; Aligiannis, N.; Skaltsounis, L. Design optimization study of the extraction of olive leaves performed with pressurized liquid extraction using response surface methodology. Sep. Purif. Technol. 2014, 122, 323–330. [CrossRef]

Delsart, C.; Cholet, C.; Ghidossi, R.; Grimi, N.; Gontier, E.; Geny, L.; Vorobiev, E.; Mietton-Peuchot, M. Effects of pulsed electric fields on cabernet sauvignon grape berries and on the characteristics of wines. Food Bioprocess Technol. 2014, 7, 424–436. [CrossRef]

Fincan, M.; DeVito, F.; Dejmek, P. Pulsed electric field treatment for solid–liquid extraction of red beetroot pigment. J. Food Eng.2004, 64, 381–388. [CrossRef]

Batra, G.; Gortzi, O.; Lalas, S.I.; Galidi, A.; Alibade, A.; Nanos, G.D. Enhanced antioxidant activity of Capsicum annuum L. and

Moringa oleifera L. extracts after encapsulation in microemulsions. ChemEngineering 2017, 1, 15. [CrossRef]

Olmo-García, L.; Bajoub, A.; Benlamaalam, S.; Hurtado-Fernández, E.; Bagur-González, M.; Chigr, M.; Mbarki, M.; Fernadéz

-Gutierréz, A.; Carrasco-Pancorbo, A. Establishing the phenolic composition of Olea europaea L. leaves from cultivars grown in morocco as a crucial step towards their subsequent exploitation. Molecules 2018, 23, 2524. [CrossRef] [PubMed]

Palmeri, R.; Parafati, L.; Trippa, D.; Siracusa, L.; Arena, E.; Restuccia, C.; Fallico, B. Addition of olive leaf extract (OLE) for producing fortified fresh pasteurized milk with an extended shelf life. Antioxidants 2019, 8, 255. [CrossRef] [PubMed]

Δημοσίευση IV

Optimization of Pulsed Electric Field as Standalone "Green" Extraction Procedure for the Recovery of High Value-Added Compounds from Fresh Olive Leaves

Vasileios M. Pappas1 , Achillia Lakka1, Dimitrios Palaiogiannis1, Vassilis Athanasiadis1 ,Eleni Bozinou1, George Ntourtoglou1,2, Dimitris P. Makris1, Vassilis G. Dourtoglou2 and Stavros I. Lalas1,*

1 **Department of Food Science & Nutrition, University of Thessaly, Terma N. Temponera Str.,GR-43100 Karditsa, Greece; vpap@uth.gr (V.M.P.); achlakka@uth.gr (A.L.); dipaleog@med.uth.gr (D.P.); vaathanasiadis@uth.gr (V.A.); empozinou@uth.gr (E.B.); gntourtoglou@uniwa.gr (G.N.); dimitrismakris@uth.gr (D.P.M.)**

2 **Department of Wine, Vine & Beverage Sciences, School of Food Science, University of West Attica, Ag. Spyridonos Str., Egaleo, GR-12243 Athens, Greece; vdourt@uniwa.gr**

ABSTRACT

Olive leaves (OLL) are reported as a source of valuable antioxidants and as an agricultural by-product/waste. Thus, a twofold objective with multi-level cost and environmental benefits arises for a "green" standalone extraction technology. This study evaluates the OLL waste valorization through maximizing OLL extracts polyphenol concentration utilizing an emerging "green" non- thermal technology, Pulsed Electric Field (PEF). It also provides further insight into the PEF assistance span for static solid-liquid extraction of OLL by choosing and fine-tuning important PEF parameters such as the extraction chamber geometry, electric field strength, pulse duration, pulse period (and frequency), and extraction duration. The produced extracts were evaluated via comparison amongst them and against extracts obtained without the application of PEF. The Folin-Ciocalteu method, high- performance liquid chromatography, and differential scanning calorimetry were used to determine the extraction efficiency. The optimal PEF contribution on the total polyphenols extractability (38% increase with a 117% increase for specific metabolites) was presented for rectangular extraction chamber, 25% *v*/*v* ethanol:water solvent, pulse duration (t_{pulse}) 2 μs, electric field strength (*E*) 0.85 kV cm^{-1}, 100 μs period (*T*), and 15 min

extraction duration ($t_{extraction}$), ascertaining a significant dependence of PEF assisting extraction performance to the parameters chosen.

Keywords: Pulsed Electric Field; fresh olive leaves; optimization; polyphenols; green; standalone

INTRODUCTION

Olive leaves (*Olea europaea* L.) (OLL) are listed as waste material from olive oil manufacturing and as aromatic or therapeutic herbs [1,2]. The global volume of OLL is estimated to be 12 Mt year^{-1} [3–5], with the majority of it produced in Europe (50%) and originating from pruning and olive oil production waste (2 Mt year^{-1}).

OLL present the most abundant agricultural waste source rich in biophenols, including phenolic acids, phenolic alcohols (hydroxytyrosol and tyrosol), flavonoids (luteolin- 7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside, luteolin-4-*O*-glucoside), and secoiridoids (oleuropein) [2,6–9]. The composition of olive leaves varies depending on the locality, seasonality, extraction solvent, and extraction procedure used. Apart from the above constituents, oleuropein is the most prominent biophenol in olive leaf extract [8,10]. Therefore, since olive leaves are reportedly a source of high amounts in bioactive compounds and an agricultural by-product, the optimization of a “green” standalone extraction technology has multi-level benefits for the environment, the green chemical engineering technology, and the end-users (pharma, medicine, food, and nutraceutical). OLL extracts production has been thoroughly investigated and various techniques and technologies are reported to be utilized for this purpose [11,12]. In particular, maceration, ultrasonic-assisted extraction, high pressure-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction are the most popular. The limitations of the above production practices originate from their thermal processing nature (causing decomposition of thermolabile compounds while having a negative environmental impact due to high energy demand), their low extraction selectivity, and their high operating costs. As a result, greener technologies (those are more energy efficient and environmentally friendly) are required to achieve improved process efficiency [13]. Furthermore, the use of fresh leaves rather than dried leaves has lately gained popularity, owing to phytochemical thermal decomposition, particularly oxidative damage to thermolabile components, during plant drying [14,15].

Pulsed Electric Field (PEF) is a relatively recent, yet emerging eco-extraction technology of biologically active compounds (BACs). PEF has minimum environmental impact since it has minimum energy requirements and a non-thermal approach. It

may be used in batch and continuous flow applications and meets the standards of green chemical engineering for long-term production systems [16]. The degree to which PEF is effective in assisting the extraction of intracellular solutes from fresh plant materials is determined from the degree to which electroporation is achieved (electrically induced formation of aqueous pores in the lipid bilayer) in a periodical and non-destructive manner for the cell under the influence of the induced transmembrane voltage by the PEF application. Electroporation occurs in such a way that components of interest migrate from the inner portion of the cell envelope to the outer part, where the solvent transports them away in solution, resulting in an increase in mass transfer and hence yield improvement for the solid-liquid extraction. The electric field strength (E), pulse shape, pulse duration (t_{pulse}),pulse period (T) or frequency (f), and total extraction duration ($t_{extraction}$) are among theparameters that must be fine-tuned when using PEF technology to improve extraction of a specific solid-liquid system [17].

PEF's influence on microorganism inactivation at high specific energy input levels [18,19], pretreatment of a variety of plant materials for downstream processes at low to moderate specific energy input levels [10,20–27], and even direct extraction of plant material are well documented [28–30]. The first reported attempt to use PEF technology as a primary extraction enhancement of high value-adding compounds from plant cell suspension cultures was by Brodelius et al. [31]. Other researchers have introduced electric field treatment for the aging acceleration of young wine, through flavor compounds extraction from wood [32,33]. Recently, Ntourtoglou et al. [34] revealed that PEF assisted extraction resulted in an increase of bitter hops acids extraction rate by 20%. Finally, Tsapou et al. [35] applied pulsed electric field (PEF) to beer wort enriched with flax seeds to fine-tune the production of phenolic aromas in beer, also by electroporation and achieved production efficiency up to 120%.

PEF technology is now being fine-tuned as the principal standalone extraction method for BACs extraction from plant material based on biomass characteristics, composition, and degree of comminution. However, there is still limited knowledge and understanding for the complex multi-parameter phenomena involved in the root cause analysis of the mechanisms that occur and affect the extraction rates of components of interest. As a result, there is lots of room for technological advancement, invention, and discovery. Usually, PEF is used as a preparative step before extraction that utilizes other techniques (such as ultrasound). In this work, the method proposed is a standalone extraction method for valuable bio-functional components that can be used in a simple, "green" and long-term manner. Furthermore, using PEF with green aqueous organic solvent mixes instead of pure water is a relatively novel method that can improve extraction yield even further. Given that each plant material exhibits a different behavior when trying to isolate one or

more of its active ingredients, it is always necessary to prove with a study that the new method is applicable. It is worth mentioning that the results or the conditions used in one study concerning a specific plant material do not necessarily apply to another.

In our previous study [36], we presented the initial results of our ongoing work on PEF and proposed this technique as a standalone extraction method of valuable bio-functional components, which can be applied in a simple "green" sustainable way for the extraction of OLL. We used an electric field of 1 kV cm^{-1} with a pulse duration of 10 or 100 μs under a period of 1000 μs for 30 min. Extraction solvents included water, ethanol, and combinations of the two.

The present study aimed to provide further insight on the PEF for a standalone solid–liquid static extraction of OLL under the target of maximizing extracts' polyphenol concentration. Towards that end, we performed a process optimization study by first choosing the best extraction chamber geometry and then by fine-tuning important PEF parameters such as the electric field strength (E), pulse shape, pulse duration (t_{pulse}), pulse period (T), and the total extraction duration ($t_{extraction}$). The average particle size, solvent type, pH, solvent to OLL ratio, and extraction temperature were kept constant throughout this study based on screening and findings from previous studies of our group [36,37]. The produced extracts were evaluated via comparison amongst them and against extracts obtained without the application of PEF. The Folin–Ciocalteu method (for total polyphenol content), high-performance liquid chromatography (HPLC), and differential scanning calorimetry (DSC) were used to assess the extraction effectiveness.

The novelty of this work lies upon the optimization of PEF, by choosing and fine-tuning important PEF parameters such as the extraction chamber geometry, electric field strength, pulse duration, pulse period (and frequency), and extraction duration, for the static solid- liquid extraction of olive leaves BACs (including the thermolabile compounds) in green solvents (pure water, pure ethanol; and their mixtures), using fresh plant material instead of dried. To the best of our knowledge there are no reports with such a holistic approach for PEF as a standalone OLL extraction. The potential of the PEF technology application in the proposed way paves the road for industrial applications (after appropriate scale-up and further fine-tuning) and new scientific discoveries.

MATERIALS AND METHODS

Chemicals

HPLC grade solvents were utilized for liquid chromatography. Acetonitrile and formic acid (99%) were purchased from Carlo Erba (Val de Reuil, France). Sodium carbonate anhydrous (99%) and gallic acid monohydrate were purchased from Penta (Prague, Czech Republic). Luteolin-7-*O*-glucoside, apigenin, rutin hydrate and oleuropein were purchased from Sigma-Aldrich (St. Louis, Burlington, MA, USA). Ethanol (99.8%) and Folin–Ciocalteu reagent were purchased from Panreac (Barcelona, Spain).

Plant Material, Handling and Sample Preparation

The OLL utilized in this study was again harvested from a 30-year-old olive tree (*Olea europaea* L., cv. Chondrolia Chalkidikis), in the Karditsa Region of Greece (at 39°21′46″ N and 21°55′05″ E, with an elevation of 108 m, according to Google Earth version 9.124.0.1, Google, Inc., Mountain View, CA, USA). After the harvesting season, the experiments were done from February 2 to February 11, 2021. The average temperature was between 9 °C and 15 °C, with an average relative humidity of 80%. Early in the morning of each experimental series day, the OLL were collected as branches and delivered to the lab 10 min later for rapid processing. After the branch removal, the leaves were completely cleaned with tap water and dried with filter paper at ambient temperature (22 °C) until no extra moisture was present on the leaves' surface. To achieve homogeneity of the pulverization outcome and minimal temperature rise, the leaves were crushed for 2 min in a blender Camry CR 4071 (Adler Europe Group ul., Warszawa, Poland) under identical shear input and batch quantities before each extraction attempt. The latter resulted in powders with an approximate average particle diameter of about 0.8 mm (d_{10} = 370 μm, d_{50} = 760 μm,

d_{90} = 1130 μm) as determined by sieve analysis.

The solvent was added to the freshly cut finely powdered OLL after grinding, and

the mixture was then placed into the PEF treatment chamber. The raw material to solvent ratio was 1:3 (*w*/*v*) in all extraction runs, with 18 g of freshly cut and finely powdered OLL and 54 mL of solvent. All experiments occurred at ambient temperature (22 °C). The suspensions were separated from the plant material, which was subsequently discarded, after each extraction. The extracts were transferred to a suitable Falcon tube and allowed for 5 min before centrifuge clarified (9164*g* for 10 min at ambient temperature). The clarified extracts were collected in Safe-Lock 2 mL Eppendorf tubes before the immediate further analysis (Y_{TP}, HPLC, and DSC). All produced PEF treated extracts were evaluated via comparison amongst them and against control extracts (obtained without the application of PEF). Triplicates of each

extraction run were performed. An infrared thermometer (GM300, Benetech, Shenzhen Jumaoyuan Science and Technology Co., Ltd., Shenzhen, China) was used to measure the temperature of the treatment chamber contents before and after each extraction run. The temperature increments owing to the treatment in all PEF assisted extraction runs never exceeded a ΔT of 1 °C. The synopsis of the plant material processing steps is presented in Figure 1.

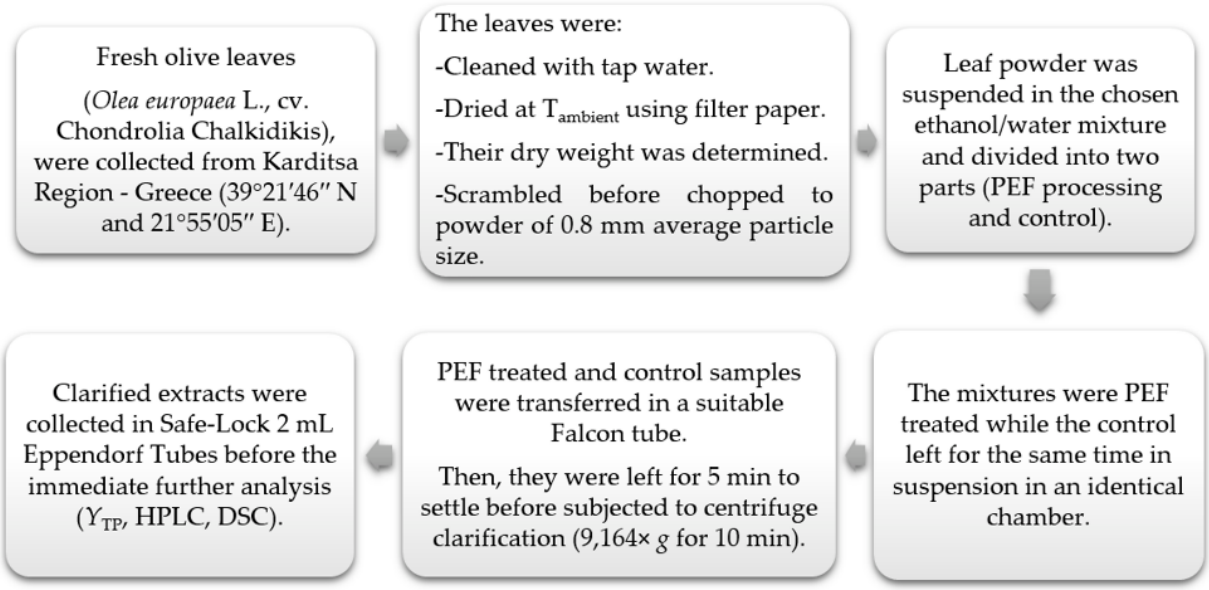


Figure 1. Plant material processing steps. Abbreviations: PEF (Pulsed Electric Field); Y_{TP} (Total Polyphenol Content); HPLC (High Performance Liquid Chromatography); DSC (Differential Scanning Calorimetry).

Dry Matter/Water Content Determination

For the determination of the water content of each batch of pulverized leaves, an adequate quantity was weighed before and after drying until constant weight, at 85 °C using an oven (Binder BD56, Bohemia, NY, USA). The following Equation (1) was then used for the calculation of the percentage of moisture and volatiles content [37]:

$$\%\ Moisture\ and\ volatiles\ content = \frac{W_{BD} - W_{AD}}{W_{BD}} \times 100$$

where W_{BD} is the weight (g) of pulverized leaves before drying, and W_{AD} is the weight(g) of pulverized leaves after drying. The leaves had a moisture and volatiles content of about 50% (*w*/*w*). Equation (2) was used to get the dry matter (g) determination for each sample [37]:

$$Dry\ matter = W_S - (W_S \times \%\ Moisture\ and\ volatiles\ content),$$

where W_S is the weight (g) of pulverized leaves without drying used as sample.

PEF System and Calculus

The PEF system used is the same with the one presented earlier by Pappas et al. [36]. It is a static bench-scale system that includes a high voltage (0.1–25 kV) power generator, a 25 MHz Function/Arbitrary Waveform Generator, a tailored electronic switch circuit (series of Insulated gate bipolar transistors—IGBTs), and two twin sets of custom-made stainless-steel treatment chambers, one rectangular and one cylindrical of similar volumes. In particular, the rectangular consists of two identical flat parallel stainless-steel plates 10 cm 10 cm separated by a "Π" shaped Teflon single piece that functions as an insulator at a regular spacing of 1 cm [36]. The cylindrical stainless-steel treatment chamber (internal diameter 3 cm and length 17 cm), including a solid stainless-steel concentric electrode (diameter 1 cm and length 17 cm), was fastened to Teflon screw caps in both ends, where the positive electrode was attached at the concentric electrode while the negative return at the outer layer of the treatment chamber. Both chambers had effective volumes equal to 80 mL.

The set of equations used for the calculation of the electric field strength (E), the total PEF treatment time ($t_{PEFtreatment}$), and the specific energy input W_{spec} (kJ kg^{-1}), are adequately described in our previous work [36]. The pulse generator provided unipolar, rectangular-shaped pulses, with pulse duration (t_{pulse}) varying between 1, 2, 5, 10, and 20 μs under a period (T) of 100, 500, and 1000 μs, for a specific number of pulses (N) defined by the extraction duration ($t_{extraction}$) and the period (T).

For the intrinsic property of conductivity, a 743 Rancimat (Metrohm UK Ltd., Cheshire WA7 1LZ, UK) was utilized, giving a measurement of 0.8 μs cm^{-1} for our solvent of choice (25% *v*/*v* EtOH:H_2O), and an average of 691 μs cm^{-1} for the extracts.

Experimental Design

The key PEF parameters were screened in depth in order to determine the best PEF parameters for the given system (plant material and solvent) in order to maximize the extracts' polyphenolic content. The permeability regulating parameters, which include field intensity (E), pulse duration (t_{pulse}), and the pulse period (T) for a specific extraction duration ($t_{extraction}$), were chosen as the major PEF parameters. Extracts of OLL treated with PEF showed greater concentrations of polyphenols at PEF pulse duration (t_{pulse}) of 10 μs, pulse period (T) of 1000 μs, electric field strength (E) of 1 kV cm^{-1}, and extraction time ($t_{extraction}$) of 30 min in aqueous ethanol, 25% *v*/*v*, as reported by Pappas et al. [36].

The study design (process optimization sections and parameters) was progress based and structured in a way that the result and conclusion from each section was adopted as input to the following section. The resulting list of experiments are presented in Table 1, and included the following sections:

Table 1. PEF process optimization study design.

Exp. Section	Exp. Series	Cell Geometry	$t_{extraxtion}$ (min)	E (kV cm^{-1})	t_{pulse} (µs)	T (µs)	N	$t_{PEFtreatment}$ (s)	Energy Input (kWh)	Specific Energy Input (kJ kg^{-1})
1	1	Rectangular	30	1	10	1000	1.80×10^{6}	18	2.52×10^{-6}	1.29×10^{-1}
	2	Rectangular	30	-	-	-	-	-	-	-
	3	Cylindrical	30	1	10	1000	1.80×10^{6}	18	2.52×10^{-6}	1.29×10^{-1}
	4	Cylindrical	30	-	-	-	-	-	-	-
2	5	Rectangular	30	1	10	1000	1.80×10^{6}	18	2.52×10^{-6}	1.29×10^{-1}
	6	Rectangular	30	0.85	10	1000	1.80×10^{6}	18	2.14×10^{-6}	1.09×10^{-1}
	7	Rectangular	30	0.7	10	1000	1.80×10^{6}	18	1.76×10^{-6}	9.00×10^{-2}
	8	Rectangular	30	-	-	-	-	-	-	-

Table 1. *Cont.*

Exp. Section	Exp. Series	Cell Geometry	$t_{extraxtion}$ (min)	E (kV cm^{-1})	t_{pulse} (µs)	T (µs)	N	$t_{PEFtreatment}$ (s)	Energy Input (kWh)	Specific Energy Input (kJ kg^{-1})
3a	9	Rectangular	30	0.85	10	1000	1.80×10^{6}	18	2.14×10^{-6}	1.09×10^{-1}
	10	Rectangular	30	0.85	5	500	3.60×10^{6}	18	2.14×10^{-6}	1.09×10^{-1}
	11	Rectangular	30	0.85	1	100	1.80×10^{7}	18	2.14×10^{-6}	1.09×10^{-1}
	12	Rectangular	30	-	-	-	-	-	-	-
3b	13	Rectangular	30	0.85	1	1000	1.80×10^{6}	2	2.14×10^{-7}	1.09×10^{-2}
	14	Rectangular	30	0.85	5	1000	1.80×10^{6}	9	1.07×10^{-6}	5.46×10^{-2}
	15	Rectangular	30	0.85	20	1000	1.80×10^{6}	36	4.28×10^{-6}	2.19×10^{-1}
	16	Rectangular	30	-	-	-	-	-	-	-
4	17	Rectangular	30	0.85	2	100	1.80×10^{7}	36	4.28×10^{-6}	2.19×10^{-1}
	18	Rectangular	15	0.85	2	100	9.00×10^{6}	18	2.14×10^{-6}	1.09×10^{-1}
	19	Rectangular	10	0.85	2	100	6.00×10^{6}	12	1.43×10^{-6}	7.29×10^{-2}
	20	Rectangular	30	-	-	-	-	-	-	-
5	21	Rectangular	15	0.85	10	1000	9.00×10^{5}	9	1.07×10^{-6}	5.46×10^{-2}
	22	Rectangular	15	0.85	2	100	9.00×10^{6}	18	2.14×10^{-6}	1.09×10^{-1}
	23	Rectangular	15	0.85	1	100	9.00×10^{6}	9	1.07×10^{-6}	5.46×10^{-2}
	24	Rectangular	15	-	-	-	-	-	-	-

Experimental Section 1. Determination of the Optimal Extraction Chamber (Cell) Geometry

As a starting point of this optimization study, the potential of whether the chamber geometry is a key parameter in the static extraction behavior was evaluated. For this reason, two different chamber geometries were tested; A cylindrical and a rectangular one (described in Section 2.4).

Experimental Section 2. Determination of the Optimal Electric Field Strength

Based on the outcome of Exp. Section 1, the optimal electric field strength was defined. Three levels of moderate intensity were utilized, namely 1, 0.85, and 0.7 kV cm^{-1}.

Experimental Section 3a. Determination of the Optimal PEF Pulse Duration

Based on the outcomes of Exp. Sections 1 and 2, the definition of the optimal pulse duration using a rectangular unipolar step-change while keeping a specific (t_{pulse}:T) analogy equal to 1:100 took place. At this section, three values were used for the t_{pulse}, namely 10, 5, and 1 μs.

Experimental Section 3b. Determination of the Optimal PEF Pulse Period

Based on the outcomes of the Exp. Sections 1 and 2, the optimal pulse duration using a rectangular unipolar step-change while altering pulse time to period analogy was defined. Here, the three sets of (t_{pulse}:T) values used were (1:1000), (5:1000), and (20:1000).

Experimental Section 4. Determination of the Optimal Extraction Time

Based on the outcomes of Sections 1, 2 and 3, followed the definition of the optimal extraction duration amongst three values, namely 30, 15, and 10 min.

Experimental Section 5. Verification

Confirmation and re-evaluation of the best cases for the optimal extraction duration.

Total Polyphenol Content of Extracts

The method was adopted by Lakka et al. [38] who employed a validated protocol (using Folin-Ciocalteu reagent) to analyze the results, which were reported as mg of gallic acid equivalents (GAE) per gram of dry weight (dw) based on the reference gallic acid calibration curve (10–80 mg L^{-1}) generated for this study. The total polyphenol yield (Y_{TP}) was calculated using the Equation (3):

$$Y_{TP}\ (\text{mg GAE g}^{-1}\ \text{of dw}) = \frac{C_{TP} \times V}{w}$$

where C_{TP} is the extract's total polyphenol concentration (mg L^{-1}), V is the volume of the extraction medium (L), and w is the plant material's dry weight (g).

High-Performance Liquid Chromatography (HPLC)

The extracts prepared during Exp. Section 5 (21, 22, 23 and 24) were analyzed using a method adopted by Kaltsa et al. [7]. A Shimadzu CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany), coupled to a Shimadzu SPD-M20A photodiode-array detector (PDA), and interfaced by Shimadzu LC solution software, was used for chromatographic studies. A Phenomenex Luna C18(2) (100 Å, 5 μm, 4.6 x 250 mm) column was employed (Phenomenex, Inc.,

Torrance, CA, USA). The temperature of the analysis was adjusted to 40 °C and the eluents used were (A) 0.5% aqueous formic acid and (B) 0.5% formic acid in acetonitrile/water (6:4). The injection volume was 20 μL and the flow rate was 1 mL min^{-1}. The gradient elution program was as follows: 100% A to 60% A in 40 min; 60% A to 50% A in 10 min; 50% A to 30% A in 10 min, which was kept constant for another 10 min. The equations of the standards calibration curves were used to accomplish quantification.

Differential Scanning Calorimetry (DSC)

After evaporating the solvents with a rotary evaporator (Laborota 4000, Heidolph, Schwabach, Germany), antioxidant activity was estimated using the DSC technique as described by Pappas et al. [36]. A Perkin Elmer Diamond DSC was used to make the measurements (PerkinElmer Inc., Shelton, CT, USA). As a purge gas, oxygen was used. In short, empty hermetically sealed pans were used as control, while 4–5 mg of each sample was placed in DSC aluminum pans with hole (1 mm in diameter) in the lids to allow the oxygen stream to reach the sample. Hold for 1 min at 40 °C, heat from 40 to 200 °C (40 °C min^{-1}), and finally heat from 200 to 580 °C (20 °C min^{-1}) were the temperature program used. The starting temperature of oxidation is determined by the onset temperature of the oxidation peak (T_{max}).

Statistical Analysis

All extraction series and spectrophotometric measurements were done in triplicate, with the average and standard deviation (SD) of three separate experiments shown. The results were statistically analyzed using Microsoft Excel 2019 (Redmond, WA, USA) software. The statistical significance (at $p < 0.05$) between mean values was determined using one-way analysis of variance (ANOVA).

RESULTS

This study focused on the PEF process optimization towards maximizing BACs content of OLL in green solvents, based on our previous work [36]. To find the optimal conditions, the variables tested were as follows; two different chamber geometries, three different electric field strengths, various pulse durations and pulse periods, and three different extraction times. A final verification section assisted in concluding the optimal conditions. The effect of the above-chosen parameters input values differentiation on the total polyphenolic composition between the control and PEF treated samples transpired via the Folin–Ciocalteu method towards extraction efficiency optimization. Further analysis of the polyphenolic profile was carried out with HPLC-PDA for the control and the samples produced under optimal conditions to determine the extraction efficiency enhancement, thus ascertain any selectivity of the

main components extracted. In addition, an estimation of the oxidation resistance of the extracts was done by utilizing the DSC technique.

Experimental Section 1 (Exp. Series 1–4)—Rectangular vs. Cylindrical Extraction Chamber

The design of the treatment chambers is critical to the development of PEF technology since they hold the sample material during PEF application and house the dischargingelectrodes. A treatment chamber is made up of two electrodes that are held in place by an insulating substance that also serves as a container for sample materials. The electrode configurations that can be used are parallel plates, parallel wires, concentric cylinders, and a rod plate [39]. Parallel plates are the most practical choice because they create a homogeneous electric field strength distribution over a large useful area. Concentric cylinders, on the other hand, provide a smooth and uniform product flow and are popular in industrial applications.

For the first section, the treatment parameters for extraction of the finely ground OLL was an electric field of 1 kV cm^{-1}, a pulse duration of 10 μs, and a pulse period of 1000 μs for a 30 min extraction duration. For the treatment chamber, two different geometries, rectangular and cylindrical were chosen. The highest percentage increase in

Y_{TP} between PEF and control samples transpired with the rectangular chamber. The results (Table 2, Exp. Series 1–4) showed that the PEF treatment into the rectangular chamber led to a 33.8% increase, while into the cylindrical utilization resulted in a 16.0%, both significant ($p < 0.05$) compared to the control samples. In particular, when PEF was applied, the Y_{TP} by the rectangular chamber appeared to be 24.98 ± 0.56 mg GAE g^{-1} dw, while the one from the cylindrical chamber reached 16.66 ± 1.55 mg GAE g^{-1} dw. Except for the lower Y_{TP} measured for the case of the cylindrical chamber versus the rectangular one, a lower percentage increase was reached when comparing the PEF treated sample in cylindrical chamber to the control. It appears that the uniformity of the field in the rectangular geometry is dominant. Thus, the rectangular chamber was chosen as the optimal geometry to continue the optimization study.

Table 2. Mean values of total polyphenol content (mg GAE g^{-1} dw) of OLL extracts.

Exp. Section	Exp. Series	Cell Geometry	$t_{extraxtion}$ (min)	E (kV cm^{-1})	t_{pulse} (μs)	T (μs)	Average Y_{TP} (mg GAE g^{-1} dw) [1]	SD	% Increase [1]	SD
1	1	Rectangular	30	1	10	1000	24.98 ^{c}	0.56	33.8 ^{B}	4.7
	2	Rectangular	30	- [2]	-	-	18.69 ^{b}	1.08	-	-
	3	Cylindrical	30	1	10	1000	16.66 ^{a}	1.55	16.0 ^{A}	5.5
	4	Cylindrical	30	-	-	-	14.42 ^{a}	2.01	-	-
-	5	Rectangular	30	1	10	1000	24.80 ^{b}	1.36	29.1 ^{A}	2.6
	6	Rectangular	30	0.85	10	1000	26.51 ^{b}	0.75	38.1 ^{B}	0.8
	7	Rectangular	30	0.7	10	1000	26.30 ^{b}	1.28	36.9 ^{B}	2
	8	Rectangular	30	-	-	-	19.20 ^{a}	0.66	-	-
3a	9	Rectangular	30	0.85	10	1000	24.69 ^{b,c}	2.24	29.8 ^{A}	2.2
	[illegible]	Rectangular	30	0.85	5	500	24.50 ^{b}	0	29.1 ^{A}	2.6
	11	Rectangular	30	0.85	1	100	24.91 ^{c}	0.18	31.2 ^{A}	2.8
	12	Rectangular	30	-	-	-	19.00 ^{a}	0.68	-	-
3b	13	Rectangular	30	0.85	1	1000	21.90 ^{b}	0.42	18.4 ^{A}	0.6
	[illegible]	Rectangular	30	0.85	5	1000	23.53 ^{c}	0.94	27.2 ^{B}	2
	15	Rectangular	30	0.85	20	1000	24.57 ^{c}	0.83	32.8 ^{C}	1.3
	16	Rectangular	30	-	-	-	18.50 ^{a}	0.45	-	-
.	17	Rectangular	30	0.85	2	100	24.75 ^{b}	0.73	35.6 ^{B}	2.7
	18	Rectangular	15	0.85	2	100	25.35 ^{b}	0.66	38.9 ^{B}	2.4
	19	Rectangular	10	0.85	2	100	17.04 ^{a}	0.21	−6.6 ^{A}	2.2
	20	Rectangular	30	-	-	-	18.30 ^{a}	1.44	-	-
-	21	Rectangular	15	0.85	10	1000	23.71 ^{b}	0.29	25.5 ^{A}	3.1
	22	Rectangular	15	0.85	2	100	25.49 ^{c}	0.88	34.9 ^{B}	0.3
	23	Rectangular	15	0.85	1	100	25.34 ^{c}	1.1	34.1 ^{B}	0.9
	24	Rectangular	15	-	-	-	18.90 ^{a}	0.69	-	-

[1] Means within rows of each Exp. Section with different superscript letters (a–c; A–C) are significantly ($p < 0.05$) different. [2] "-" denotes no values for control samples (no PEF applied).

Experimental Section 2 (Exp. Series 5–8)—Optimal Electric Field Strength

All the cells in the sample are exposed to the same electric field in uniform electric field chambers, which is beneficial for electroporation. If the field strength is enough and close to the optimal value, high intracellular compound extraction yields are feasible. However, given that optimum extraction yields can fall significantly above or below the optimum field strength, the optimum value for the electric field strength must always be determined through structured experimental design.

Based on the literature [40], it was decided to screen the electric field for an optimal effect on the extraction of bioactive compounds from OLL at the range of 0.7 to 1 kV cm^{-1}, keeping the level of specific energy input bellow 5 kJ kg^{-1}. Thus, for this optimization section (Exp. Section 2), three different input values for the electric field strength were tested, namely 1, 0.85, and 0.7 kV cm^{-1}. The field strength of 1 kV cm^{-1} resulted in an increase of 29.1% in Y_{TP}. In particular, the results (Table 2, Exp. Series 5–8) showed that the specific PEF sample gave a Y_{TP} of 24.80 ± 1.36 mg GAE g^{-1} dw while the control sample 19.20 ± 0.66 mg GAE g^{-1} dw. The application of field strengths of 0.85 and 0.7 kV cm^{-1} resulted in higher percentage significant ($p < 0.05$) increases (38.1% and 36.9%, respectively). The highest increase was observed for the case of 0.85 kV cm^{-1} field strength and is in line with previous studies. Although, it was

not significantly different than that of 0.7 kV cm^{-1}, it was selected to continue the optimization study.

Experimental Section 3a and 3b—Optimal PEF Pulse Duration and Period (Exp. Series 9–12 and 13–16)

For Exp. Section 3 of the optimization study, different pulse durations or periods were examined, thus altering the cell membrane relaxation time or the specific energy applied to the sample towards revealing the best combination for the OLL extraction. For this section, based on the outcome of Exp. Sections 1 and 2, the starting point was the rectangular chamber and electric field strength of 0.85 kV cm^{-1} for an extraction duration of 30 min. The targeted research inquiry of this optimization section was twofold. For the first part, the changes of t_{pulse} and T followed a constant ratio of 1:100, while for the second part, we experimented with different t_{pulse} under a fixed T.

For Exp. Section 3a (Table 2, Exp. Series 9–12), the highest percentage increase between PEF and the control sample was achieved when a pulse duration of 1 μs and a pulse period of 100 μs (31.2%) was applied. In particular, the Y_{TP} for the extract produced with t_{pulse} 1 μs and T 100 μs was 24.91 ± 0.18 mg GAE g^{-1} dw while for the control, it was 19.00 0.68 mg GAE g^{-1} dw. Similar increases transpired by applying t_{pulse} 10 μs with T 1000 μs and t_{pulse} 5 μs with T 500 μs, which were 29.8% and 29.1%, respectively.

For Exp. Section 3b (Table 2, Exp. Series 13–16), the highest percentage increase was obtained with t_{pulse} 20 μs and T 1000 μs, namely 32.8% (significant at $p < 0.05$). In particular, the PEF sample resulted in a Y_{TP} of 24.57 ± 0.83 mg GAE g^{-1} dw, while for the control, it was 18.50 ± 0.45 mg GAE g^{-1} dw. For the rest of the PEF conditions tested, namely t_{pulse} 1 μs with T 1000 μs and t_{pulse} 5 μs with T 1000 μs, lower but significant ($p < 0.05$) increases were observed, particularly 18.4% and 27.2%, respectively.

The outcome from Exp. Sections 3a and 3b indicated the preference for a short pulse period and specifically the set of t_{pulse} of 2 μs and T of 100 μs. Therefore, these conditions were selected for the next step of the optimization study.

Experimental Section 4 (Exp. Series 17–20)—Optimal Extraction Time

In Exp. Section 4 (Table 2, Exp. Series 17–20), the effect of the extraction time was quantified as the last parameter of choice for the completion of the PEF assisted OLL extraction optimization. An electric field strength of 0.85 kV cm^{-1}, with a t_{pulse} of 2 μs and a T of 100 μs, was applied for three different extraction times, namely 30, 15, and 10 min. The extraction time of 30 min led to an increase

of 35.6% (significant at $p < 0.05$). However, for 15 min treatment duration, an even higher increase (38.9%) was observed ($p < 0.05$). Possibly the exposure of the samples to the air for more time (30 min) increased the oxidation of some compounds. In particular, the Y_{TP} for the control sample was 18.30 ± 1.44 mg GAE g^{-1} dw while for the PEF treated extract, Y_{TP} reached a 25.35 ± 0.66 mg GAE g^{-1} dw. In contrast to the above trend, the 10 min treatment time proved to be insufficient, having a not significant difference in contrast to the control sample concerning Y_{TP}.

From the outcome of Exp. Section 4, the extraction duration of choice for the final verification section of our study was 15 min, half from the initially applied. Such a result has several economical and practical benefits, and it should be utilized at an industrial scale for obvious reasons.

Experimental Section 5 (Exp. Series 21–24)—Verification Section

Finally, in Exp. Section 5 (Table 2, Exp. Series 21–24), a verification check was performed for the optimal cases found in the previous sections with the difference of applying 15 min instead of 30 min for the extraction duration. As shown in Table 2, the results appear to follow the findings when the treatment time was 30 min. In particular, the highest increase was 34.9% and transpired using t_{pulse} of 2 μs and T of 100 μs. A similar increment (34.1%) resulted by applying t_{pulse} of 1 μs and T of 100 μs. The lowest increase was 25.5% when t_{pulse} of 10 μs and T of 1000 μs were utilized. The percentage increases were not significant between Exp. Series 22 and 23.

Characterization of the Extracts Using HPLC—Polyphenolic Composition of Exp. Series 21–24

The main components of OLL found during this study are following the literature [6–9,36,41,42]. In specific, the predominant constituents are oleuropein and luteolin- 7-*O*-glucoside. Additionally, luteolin's related substances are in lower amounts, as well apigenin-7-*O*-rutinoside and quercetin-3-*O*-rutinoside. Seven main compounds were revealed from the chromatogram at 345 nm (Figure 2).

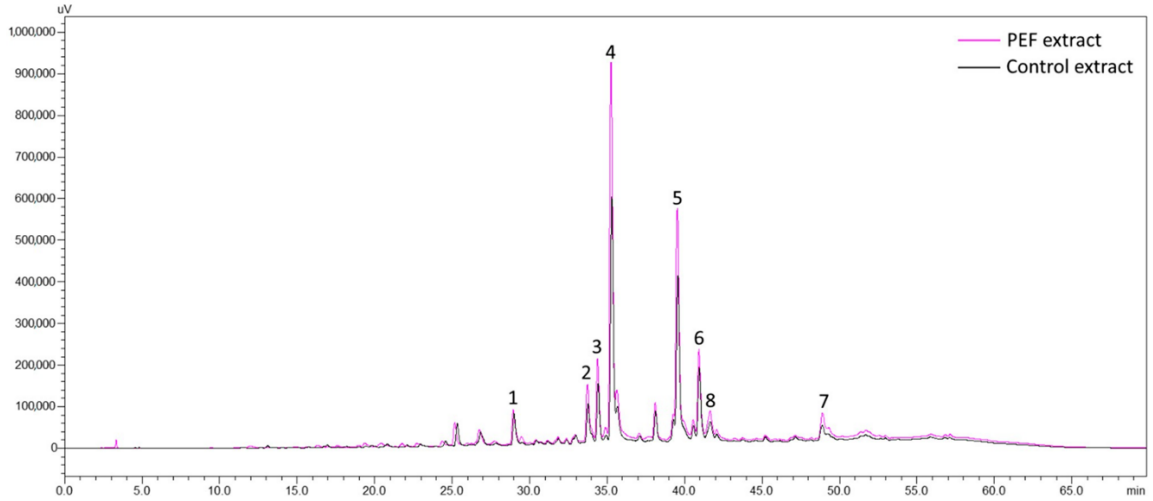


Figure 2. Overlay of chromatograms of PEF and control extracts with t_{pulse} 10 µs and T 1000 µs. Peak 1: Luteolin diglucoside; Peak 2: Quercetin-3-*O*-rutinoside; Peak 3: Luteolin rutinoside; Peak 4: Luteolin-7-*O*-glucoside; Peak 5: Apigenin-7-*O*-rutinoside; Peak 6: Luteolin-3′-*O*-glucoside; Peak 7: Luteolin aglycone; Peak 8: Oleuropein.

The peaks 2 and 4 with a λ_{max} at 349 nm and 345 nm were identified according to their retention time, absorption spectrum, and their corresponding reference substances as quercetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside, respectively. From our previous work [7], peaks 5 and 6 were tentatively identified (by LC-DAD-MS) as apigenin-7-*O*- rutinoside and luteolin-3′-*O*-glucoside, respectively. Due to the lack of corresponding reference substances, peaks 1, 3, and 7 could not be identified, even though according to lit- erature [6,7] and their similar UV-Vis spectrum to luteolin-7-*O*-glucoside, it is believed to be related substances of luteolin. In specific, peak 1 is believed to be luteolin diglucoside which Mylonaki et al. [9] and Herrero et al. [10] identified as luteolin diglucoside eluting barely be- fore quercetin-3-*O*-rutinoside with a λ_{max} at 331 nm, just like peak 1 in Figure 2 with a λ_{max} at 333 nm. From the findings of Herrero et al. [6], luteolin rutinoside is eluted with a λ_{max} at 340 nm between quercetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside. Thus, it is believed that peak 3 is luteolin rutinoside since the chromatogram follows identical elution order with a λ_{max} at 345 nm. Peak 8 with a λ_{max} at 280 nm was identified as oleuropein based on its retention time and absorption spectrum with the corresponding reference substance. The control sample extracts for the main compounds achieved amounts 0.76 ± 0.03 mg g^{-1} dw for luteolin-7-*O*-glucoside and 0.65 ± 0.04 mg g^{-1} dw for oleuropein (Table 3). Lower amounts were reached by quercetin-3-*O*-rutinoside, apigenin-7-*O*-rutinoside, and luteolin- 3′-*O*-glucoside (0.16 ± 0.01, 0.25 ± 0.01, and 0.25 ± 0.03 mg g^{-1} dw, respectively).

Table 3. Major compounds concentration (mg g^{-1} dw) of OLL extracts prepared with 25% aqueous ethanol for extraction duration of 15 min.

Exp. Series	Concentration Parameters	Peak 1 [1]	Quercetin-3-*O*-Rutinoside	Peak 3 [1]	Luteolin-7-*O*-Glucoside	Apigenin-7-*O*-Rutinoside [2]	Luteolin-3′-*O*-Glucoside [1]	Oleuropein	Peak 7 [1]
21	Average [3]	0.14 [a]	0.25 [b]	0.27 [b]	1.30 [c]	0.39 [c]	0.31 [b]	1.12 [c]	0.09 [b]
	SD	0.02	0.03	0.01	0.04	0.01	0.02	0.06	0
	% Increase [3]	26.87 [B]	55.87 [A]	42.11 [B]	70.31 [C]	56.06 [B]	24.56 [A]	72.36 [B]	42.86 [B]
	SD	6.68	9.03	5.26	0.72	2.24	7	1.38	12.37
22	Average	0.13 [a]	0.24 [b]	0.20 [a]	1.08 [b]	0.36 [b,c]	0.28 [a]	1.41 [d]	0.07 [a]
	SD	0.02	0.01	0.01	0.05	0.02	0.01	0.07	0
	% Increase	17.73 [A,B]	50.13 [A]	6.14 [A]	41.02 [A]	43.94 [A]	10.73 [A]	117.58 [C]	11.11 [A]
	SD	7.51	3.14	4.02	1.6	2.24	9.52	3.46	9.62
23	Average	0.13 [a]	0.24 [b]	0.28 [b]	1.24 [c]	0.37 [b]	0.31 [b]	1.01 [b]	0.10 [b]
	SD	0.01	0.02	0.01	0.03	0	0.02	0.03	0.01
	% Increase	18.28 [A]	49.87 [A]	47.37 [B]	62.48 [B]	48.16 [A,B]	25.32 [A]	55.59 [A]	57.94 [B]
	SD	1.67	3.14	5.26	1.5	5.93	8.2	4.97	8.36
24	Average	0.11 [a]	0.16 [a]	0.19 [a]	0.76 [a]	0.25 [a]	0.25 [a]	0.65 [a]	0.06 [a]
	SD	0.01	0.01	0	0.03	0.01	0.03	0.04	0.01
	% Increase	- [4]	-	-	-	-	-	-	-

[1] Luteolin-3′-*O*-glucoside as well as peaks 1, 3 and 7 were quantified as luteolin-7-*O*-glucoside. [2] Apigenin-7-*O*-rutinoside was quantified as apigenin. [3] Means within each column (compound) with different superscript letters (a–c; A–C) are significantly ($p < 0.05$) different. [4] "-" denotes no values for control samples (no PEF applied).

It is recognized that the main factors that rule the multitude and the levels of the components detected are the solvent choice, the extraction method, the seasonality, and the locality [43–45]. The concentrations of the main identified compounds of OLL extracts between the control sample and three conditions of PEF with 15 min $t_{extraction}$ were evaluated to define the PEF treatment effect and especially how different combinations of pulse durations and periods change the polyphenolic composition in the extracts (Table 3, Figure 3).

In specific, the PEF conditions with t_{pulse} 10 μs and T 1000 μs, t_{pulse} 2 μs and T 100 μs and t_{pulse} 1 μs and T 100 μs were examined (for Exp. Series 21–24), while the other PEF parameters were unchanged. In most cases, all the above PEF treatment conditions have

shown significant enhancements to the amounts of the tested constituents, which led to an increase up to 117.58%, proving that a disintegration effect on cell membranes of OLL was sufficiently successful even for shorter t_{pulse} and T. The PEF condition with t_{pulse} 10 μs and T 1000 μs (Exp. Series 21) reached higher percentage increases than the other two conditions for five of the eight components examined. In specific, for peak 1, quercetin-3-*O*-rutinoside, luteolin-7-*O*-glucoside, apigenin-7-*O*-rutinoside and luteolin-3′- *O*-glucoside was 26.87%, 55.87%, 70.31%, 56.06% and 24.56%, respectively. The rest of the compounds, namely peak 3, oleuropein, and peak 7, ranged from 42.11% to 72.36%. As the PEF treatment with t_{pulse} 1 μs and T 100 μs (Exp. Series 23) is concerned, it led to a higher increase for peak 3 and peak 7 (47.37% and 57.94%, respectively), while the increment for the other constituents ranged from 18.28% to 62.48%. The highest increase for oleuropein was 117.58% and achieved by the PEF condition with t_{pulse} 2 μs and T 100 μs (Exp. Series

22). For this condition, the increment for the rest compounds ranged from 6.14% to 50.13%.

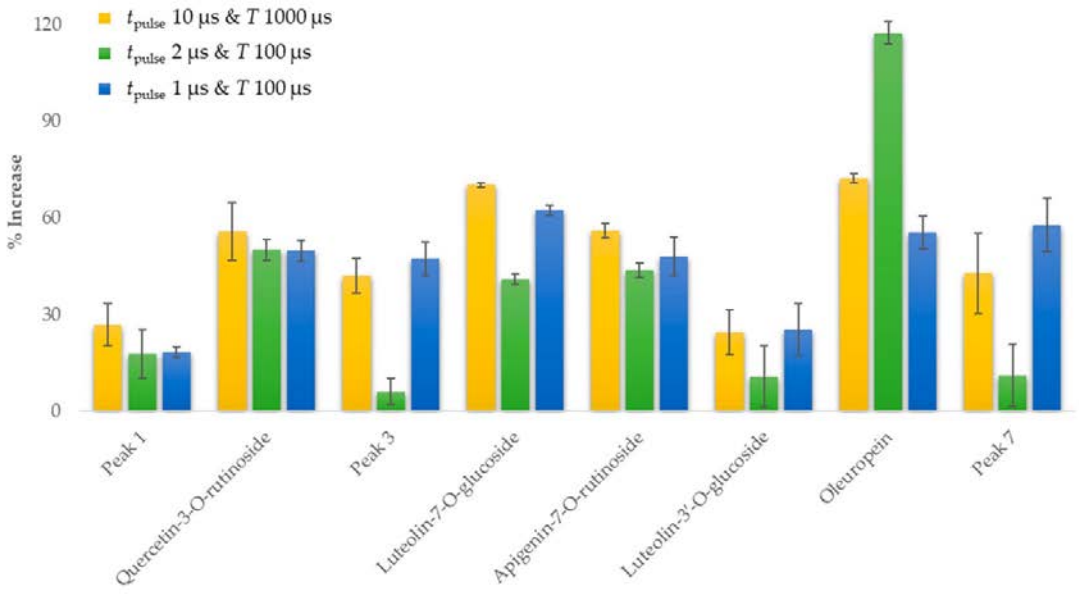


Figure 3. Percentage increases for the main compounds on the three best combinations of t_{pulse} and T.

For the main compound of OLL, namely luteolin-7-*O*-glucoside, Palmeri et al. [42] achieved an amount of 0.82 mg g^{-1} dw by applying an extraction with water as solvent, high temperature, and a high liquid to solid ratio of 20:1 mL g^{-1}. In our work, the same metabolite reached 1.30 ± 0.04 mg g^{-1} dw for the PEF condition with t_{pulse} 10 µs and T 1000 µs (Exp. Series 21). The significance of this result is based on that a higher amount was gained from a nonthermal effective green extraction method, using a low liquid to solid ratio (3:1). Furthermore, the low percentage (25%) of "green" solvent (EtOH) used, minimizes the cost of its recovery and recycling procedure in the final product and, thus, eliminate the environmental limitations. Additionally, this quantity of luteolin-7-*O*- glucoside appears two times higher than that we have previously reported [36], possibly because of the optimization procedure followed during the work and the different time of collection of OLL (variance in plant material). Concerning the secondary components, quercetin-3-*O*-rutinoside, apigenin-7-*O*-rutinoside, and luteolin-3′-*O*-glucoside, the PEF treatment led to amounts near 0.3 mg g^{-1} dw, where similar concentrations were reached in recent studies [7,42] where high liquid to solid ratios and high energy input to the sample were applied. Finally, for oleuropein, Cifa et al. [46] reached 3.1 mg g^{-1} dw, using a similar percentage of ethanol (30%), somewhat higher liquid to solid ratio (5:1), and ultrasound application for 120 min (energy input range of 12 x 10^3 kJ kg^{-1}). The highest concentration of oleuropein, succeeded with PEF treatment in our work,

was 1.41 ± 0.07 mg g^{-1} dw in a much shorter extraction time (15 min) and much lower energy input to the sample (range of 0.1 kJ kg^{-1}). However, the results of the above authors are not directly comparable with those of the present work since they have used leaves of a different *O. europaea* L. variety which was also cultivated in different agroclimatic conditions.

Differential Scanning Calorimetry (DSC)

Antioxidant oxidative stability can be determined using the DSC technique [47]. The estimated temperature at the start of the oxidation process based on observations taken during the incubation period is used to assess this stability. As a result, DSC may be used to deduce oxidation kinetic parameters from the thermographic curves that provide the temperature of the extrapolated initiation of the thermo-oxidation process [48]. T_{max}, in particular, is the thermographic curve's highest oxidation peak. The greater the T_{max} value, the higher the sample's resistance. DSC was used to measure the exothermic peaks of the extracts in this study (Table 4). According to the results, the T_{max} was found to be directly related to the total polyphenol content of each sample. The samples of Exp. Series 6 (pulse duration: 10 μs, pulse period: 1000 μs, electric field: 0.85 kV cm^{-1}, time of extraction: 30 min) achieved the highest oxidation peak (T_{max}) of 488 °C (significant at $p < 0.05$), as well as the highest percentage increase (significant at $p < 0.05$) in comparison to the control sample (Exp. Series 8).

Table 4. DSC results on the oxidation temperature (T_{max}) of the various samples.

Exp. Section	Exp. Series	PEF Treated Extract		Exp. Series	Control Extract		% Increase [1]	SD
		Average Oxidation Temperature (°C) [1]	SD		Average Oxidation Temperature (°C) [1]	SD		
1	1	476 [a]	1	2	415 [b]	1	14.78 [B]	0.81
	3	411 [c]	2	4	401 [c]	1	2.49 [A]	0.24
2	5	475 [a]	1	8	418 [d]	2	13.63 [A]	0.89
	6	488 [b]	2				16.82 [B]	0.76
	7	484 [c]	1				15.95 [B]	0.53
3a	9	473 [a]	1	12	416 [c]	1	13.70 [A]	0.67
	10	472 [a]	1				13.46 [A]	0.69
	11	477 [b]	1				14.66 [A]	0.56
3b	13	459 [a]	2	16	414 [d]	2	10.95 [A]	0.19
	14	468 [b]	1				13.04 [B]	0.18
	15	474 [c]	1				14.57 [C]	0.09
4	17	475 [a]	2	20	414 [d]	2	14.73 [B]	0.65
	18	480 [b]	1				15.94 [B]	0.89
	19	412 [c]	1				−0.48 [A]	0.24
5	21	469 [a]	2	24	416 [c]	1	12.74 [A]	0.21
	22	482 [b]	1				15.87 [B]	0.24
	23	480 [b]	2				15.38 [B]	0.2

[1] Means within rows (Exp. Sections) with different superscript letters (a–d; A–C) are significantly ($p < 0.05$) different.

DISCUSSION

The novelty of this study was to deal with optimizing PEF technology as a standalone solid-liquid extraction method for bioactive constituents from freshly cut OLL, under the goal of developing and proposing a method that would replace the conventional extraction techniques by substantially reducing the use of organic solvents and the energy input towards a more efficient, effective, and environmentally friendly polyphenolic compound isolation technique in an economically feasible way.

Overall, PEF enables a higher rate of diffusivity by triggering cell permeabilization changes and, thus, forcing the migration of intracellular components of interest to a solution. Our study design evaluated all the critical parameters affecting the extraction yield of the bioactive compounds comprehensively. The results indicated a significant increase in the total polyphenolic content of the obtained extracts produced using a "green" solvent mixture under fine-tuned PEF conditions.

The amount and nature of the extracted polyphenols depended on the chamber geometry, the applied electric field, the pulse duration and period, and the treatment time; allowing for interesting quantitative and qualitative conclusions over the correlation of the above parameters with the electroporation optimal energy range for the specific plant material cells, the achieved extraction yield and the structure of the extracted metabolites. The optimal detected PEF contribution on the total polyphenols extractability (38% increase) and constituents of interest for the food, pharma and cosmetic industry (up to 117% increase for specific metabolites) transpired for a rectangular-shaped extraction chamber and 25% *v*/*v* aqueous ethanol solvent choice using a pulse duration (t_{pulse}) of 2 μs under 0.85 kV cm^{-1} electric field strength (E), and a period (T) of 100 μs for a 15 min extraction duration ($t_{extraction}$) ascertaining a significant dependence of PEF assisting extraction performance to the parameters chosen in this study.

Comparing to our previous study [36], for the same raw material (same tree but different season), we reached levels of Y_{TP} that resulted from much higher EtOH content solvents (75% EtOH:H_2O) in half the extraction duration. In particular, during our previous study, we reached 31.45 mg GAE g^{-1} dw with 75% EtOH, $t_{extraction}$ of 30 min, and electric field strength of 1 kV cm^{-1}, while, in this study, a similar yield (25.49 mg GAE g^{-1} dw) transpired after the optimization of the PEF assisted extraction procedure with only 25% EtOH, 15 min $t_{extraction}$, and 0.85 kV cm^{-1} electric field strength. Thus, the achievement is both energy and cost-effective, reducing the cost of the whole process while increasing the environmental friendliness of the process.

From the comparative difference of compound concentration percentage increment on each PEF condition, it appears that PEF conditions (such as t_{pulse} and T) affect the extraction rate of intracellular components in a nonlinear manner, demonstrating the

selectivity of this extraction method. The latter claim is strengthened by the observations and outcome of our previous study [36], where we noticed that t_{pulse} affected the extraction rate of identified components, allowing for the selective extraction of distinct OLL molecules. Given that selective extraction is a difficult, time-consuming, and energy-intensive technique, this discovery is critical.

The molecular structure and therefore size, changes in cell membrane breakdown (such as pore size), as well as the solubility of extracted components and the solvent's polarity, are all possible causes of this selectivity [48,49]. With the exception of oleuropein, the t_{pulse} of 10 μs achieved higher or satisfactory increases (no significant difference) for all substances apart from phenolic glycosides, where the molecule usually comprises one or two sugar units bound to a flavone-backbone (quercetin). Because oleuropein's molecular structure differs from other phenolic compounds in its lack of a flavone backbone and its smaller size, the shorter t_{pulse} of 2 μs produced considerably ($p < 0.05$) superior outcomes. Additionally, the different solubility of each component is a crucial factor in PEF treated samples. The solubility of the various components was reported in our previous work [36]. In brief, quercetin-3-*O*-rutinoside has a much lower water solubility than the other compounds, while ethanol is an excellent solvent for oleuropein (all other glycosides are less soluble in it).

Larger molecules tend to require longer continuous pulse duration for selective extraction. As a result, the key to their optimum selective extraction is a combination of molecular size and solubility.

The results showed that the T_{max} was directly related to the total polyphenol content of each sample when using DSC to determine the higher oxidation resistance.

CONCLUSIONS

Based on our findings, the PEF application boosted the performance of conventional static solid–liquid extraction of specific bioactive compounds from fresh olive leaves in an eco-friendly way utilizing green solvents. Even though industrial limitations can originate from the static nature of the standalone extraction optimization proposed technology for continuous flow industrial applications, PEF presents an excellent potential for green selective extraction of polyphenolic compounds from OLL. PEF assisted extraction technology can revitalize functional food manufacturing in a sustainable fashion, generating high- quality products enriched with BACs that have several public health benefits, depending on the biomass qualities, availability, composition, and degree of comminution.

Complementary work is strongly advisable to include the solvent, pH and, polarity effect in the PEF outcome towards maximizing polyphenols concentration. Future

work should also focus on further optimization of PEF process parameters to further validate and maximize the selective polyphenols concentration. Another area of future research interest is the influence of chamber content conductivity in the PEF effect.

Author Contributions: Conceptualization, V.M.P., S.I.L. and V.G.D.; Methodology, V.M.P., G.N. and A.L.; Validation, A.L.; Formal Analysis, A.L., D.P., E.B. and V.A.; Investigation, V.M.P., A.L., D.P., V.A., E.B. and G.N.; Resources, S.I.L.; Data Curation, V.M.P., A.L., V.A. and D.P.; Writing—Original Draft Preparation, V.M.P., A.L. and D.P.; Writing—Review and Editing, S.I.L., V.G.D., V.M.P., V.A. and E.B.; Supervision, S.I.L., V.G.D. and D.P.M.; Project Administration, S.I.L.; Funding Acquisition, S.I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH- CREATE- INNOVATE (project code: T1EDK-03762).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Sahin, S.; Bilgin, M. Olive tree (Olea europaea L.) leaf as a waste by-product of table olive and olive oil industry: A review. J. Sci. Food Agric. 2018, 98, 1271–1279. [CrossRef] [PubMed]

Toledano, A.; Alegria, I.; Labidi, J. Biorefining of olive tree (Olea europea) pruning. Biomass Bioenergy 2013, 59, 503–511. [CrossRef]

Khdair, A.; Abu-Rumman, G. Sustainable Environmental Management and Valorization Options for Olive Mill Byproducts in the Middle East and North Africa (MENA) Region. Processes 2020, 8, 671. [CrossRef]

Romero, I.; Ruiz, E.; Castro, E.; Moya, M. Acid hydrolysis of olive tree biomass. Chem. Eng. Res. Des. 2010, 88, 633–664. [CrossRef]

FAOSTAT. Food and Agriculture Organization Database. Available online: http://www.fao.org/home/search/en/?q=Olive%20 mill%20wastes%20production (accessed on 4 August 2021).

Herrero, M.; Temirzoda, T.N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. J. Chromatogr. A 2011, 1218, 7511–7520. [CrossRef] [PubMed]

Kaltsa, O.; Grigorakis, S.; Lakka, A.; Bozinou, E.; Lalas, S.; Makris, D. Green valorization of olive leaves to produce polyphenol- enriched extracts using an environmentally benign deep eutectic solvent. AgriEngineering 2020, 2, 14. [CrossRef]

Lalas, S.; Athanasiadis, V.; Gortzi, O.; Bounitsi, M.; Giovanoudis, I.; Tsaknis, J.; Bogiatzis, F. Enrichment of table olives with polyphenols extracted from olive leaves. Food Chem. 2011, 127, 1521–1525. [CrossRef]

Mylonaki, S.; Kiassos, E.; Makris, D.; Kefalas, P. Optimization of the extraction of olive (Olea europaea) leaf phenolics using water/ethanol-based solvent systems and RSM. Anal. Bioanal. Chem. 2008, 392, 977–985. [CrossRef]

Briante, R.; La Cara, F.; Febbraio, F.; Patumi, M.; Nucci, R. Bioactive derivatives from oleuropein by a biotransformation on Olea europaea leaf extracts. J. Biotechnol. 2002, 93, 109–119. [CrossRef]

Mahugo Santana, C.; Sosa Ferrera, Z.; Esther Torres Padrón, M.; Juan Santana Rodríguez, J. Methodologies for the extraction of phenolic compounds from environmental samples: New approaches. Molecules 2009, 14, 298. [CrossRef]

Otero, D.M.; Oliveira, F.M.; Lorini, A.; Antunes, B.F.; Oliveira, R.M.; Zambiazi, R.C. Oleuropein: Methods for extraction, purifying and applying. Rev. Ceres 2020, 67, 315–329. [CrossRef]

Sosa Ferrera, Z.; Padrón Sanz, C.; Mahugo Santana, C.; Santana Rodríguez, J.J. The use of micellar systems in the extraction and pre-concentration of organic pollutants in environmental samples. TrAC Trend. Anal. Chem. 2004, 23, 469–479. [CrossRef]

Asami, D.K.; Hong, Y.J.; Barrett, D.M.; Mitchell, A.E. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. J. Agric. Food Chem. 2003, 51, 1237–1241. [CrossRef]

Mueller-Harvey, I. Analysis of hydrolysable tannins. Anim. Feed Sci. Technol. 2001, 91, 3–20. [CrossRef]

Chemat, F.; Rombaut, N.; Meullemiestre, A.; Turk, M.; Perino, S.; Fabiano-Tixier, A.-S.; Abert-Vian, M. Review of green food processing techniques. Preservation, transformation, and extraction. Innov. Food Sci. Emerg. 2017, 41, 357–377. [CrossRef]

Ben Farhat, M.; Chaouch-Hamada, R.; Sotomayor, J.A.; Landoulsi, A.; Jordan, M.J. Antioxidant potential of *Salvia officinalis* L. residues as affected by the harvesting time. Ind. Crops Prod. 2014, 54, 78–85. [CrossRef]

Álvarez, I.; Pagán, R.; Condon, S.; Raso, J. The influence of process parameters for the inactivation of Listeria monocytogenes by pulsed electric fields. Int. J. Food Microbiol. 2003, 87, 87–95. [CrossRef]

Grahl, T.; Märkl, H. Killing of microorganisms by pulsed electric fields. Appl. Microbiol. Biotechnol. 1996, 45, 148–157. [CrossRef]

Bobinaite˙, R.; Pataro, G.; Lamanauskas, N.; Šatkauskas, S.; Viškelis, P.; Ferrari, G. Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products. J. Food Sci. Technol. 2015, 52, 5898–5905. [CrossRef]

Carullo, D.; Pataro, G.; Ferrari, G. Effect of PEF pre-treatment and extraction temperature on the recovery of carotenoids from tomato wastes. Chem. Engineer. Trans. 2019, 75, 139–144. [CrossRef]

Hendrawan, Y.; Larasati, R.; Wibisono, Y.; Umam, C.; Sutan, S.; Hawa, L. Extraction of phenol and antioxidant compounds from kepok banana skin with PEF pre-treatment. IOP Conf. Ser. Earth Environ. Sci. 2019, 305, 012065. [CrossRef]

Luengo, E.; Álvarez, I.; Raso, J. Improving carotenoid extraction from tomato waste by pulsed electric fields. Front. Nutr. 2014, 1, 12.

[CrossRef] [PubMed]

Martín, B.; Tylewicz, U.; Verardo, V.; Pasini, F.; Caravaca, A.M.G.; Caboni, M.; Dalla Rosa, M. Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers' spent grains. Innov. Food Sci. Emerg. 2020, 64, 102402. [CrossRef]

Pataro, G.; Carullo, D.; Siddique, A.; Falcone, M.; Donsì, F.; Ferrari, G. Improved extractability of carotenoids from tomato peels as side benefits of PEF treatment of tomato fruit for more energy-efficient steam-assisted peeling. J. Food Eng. 2018, 233, 65–73. [CrossRef]

Sukardi, S.; Purwaningsih, I.; Sita, P. Extraction of phenolic compounds from basil (Ocimum americanum L.) leaves with pretreat- ment using pulsed electric field (PEF). IOP Conf. Ser. Earth Environ. Sci. 2020, 475, 012056. [CrossRef]

Tzima, K.; Brunton, N.P.; Lyng, J.G.; Frontuto, D.; Rai, D.K. The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products. Innov. Food Sci. Emerg. Technol. 2021, 69, 102644. [CrossRef]

Bozinou, E.; Karageorgou, I.; Batra, G.; Dourtoglou, V.; Lalas, S. Pulsed electric field extraction and antioxidant activity determination of Moringa oleifera dry leaves: A comparative study with other extraction techniques. Beverages 2019, 5, 8. [CrossRef]

Lakka, A.; Bozinou, E.; Makris, D.P.; Lalas, S.I. Evaluation of Pulsed Electric Field polyphenol extraction from Vitis vinifera, Sideritis scardica and Crocus sativus. Chemengineering 2021, 5, 25. [CrossRef]

Lakka, A.; Bozinou, E.; Stavropoulos, G.; Samanidis, I.; Athanasiadis, V.; Dourtoglou, V.G.; Makris, D.P.; Lalas, S.I. Enhancement of polyphenols recovery from Rosa canina, Calendula officinalis and Castanea sativa using Pulsed Electric Field. Beverages 2021, 7, 63. [CrossRef]

Brodelius, P.E.; Funk, C.; Shillito, R.D. Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored secondary products. Plant Cell Rep. 1988, 7, 186–188. [CrossRef]

Drosou, F.; Yang, E.; Marinea, M.; Dourtoglou, T.; Chatzilazarou, A.; Dourtoglou, V. An assessment of potential applications with pulsed electric field in wines. BIO Web Conf. 2017, 9, 02010. [CrossRef]

Zeng, X.A.; Yu, S.; Zhang, L.; Chen, X. The effects of AC electric field on wine maturation. Innov. Food Sci. Emerg. 2008, 9, 463–468.

[CrossRef]

Ntourtoglou, G.; Tsapou, E.A.; Drosou, F.; Bozinou, E.; Lalas, S.; Tataridis, P.; Dourtoglou, V. Pulsed electric field extraction of α and β-acids from pellets of Humulus lupulus (hop). Front. Bioeng. Biotechnol. 2020, 8, 297. [CrossRef]

Tsapou, E.A.; Ntourtoglou, G.; Drosou, F.; Tataridis, P.; Dourtoglou, T.; Lalas, S.; Dourtoglou, V. In situ creation of the natural phenolic aromas of beer: A pulsed electric field applied to wort-enriched flax seeds. Front. Bioeng. Biotechnol. 2020, 8, 1219. [CrossRef]

Pappas, V.M.; Lakka, A.; Palaiogiannis, D.; Bozinou, E.; Ntourtoglou, G.; Batra, G.; Athanasiadis, V.; Makris, D.P.; Dourtoglou, V.G.; Lalas, S.I. Use of Pulsed Electric Field as a low-temperature and high-performance “green” extraction technique for the recovery of high added value compounds from olive leaves. Beverages 2021, 7, 45. [CrossRef]

Athanasiadis, V.; Lakka, A.; Palaiogiannis, D.; Pappas, V.M.; Bozinou, E.; Ntourtoglou, G.; Makris, D.P.; Dourtoglou, V.G.; Lalas, S.I. Pulsed Electric Field and *Salvia officinalis* L. leaves: A successful combination for the extraction of high added value compounds. Foods 2021, 10, 2014. [CrossRef]

Lakka, A.; Grigorakis, S.; Karageorgou, I.; Batra, G.; Kaltsa, O.; Bozinou, E.; Lalas, S.; Makris, D.P. Saffron processing wastes as a bioresource of high-value added compounds: Development of a green extraction process for polyphenol recovery using a natural deep eutectic solvent. Antioxidants 2019, 8, 586. [CrossRef]

Hoffman, G.A. Cells in Electric Fields: Physical and Practical Electronic Aspects of Electro Cell Fusion and Electroporation. In Electroporation and Electrofusion in Cell Biology, 1st ed.; Jordan, C.A., Neumann, E., Sowers, A.E., Eds.; Plenum Press: New York, NY, USA, 1989; Volume 1, pp. 389–407. [CrossRef]

Donsì, F.; Ferrari, G.; Pataro, G. Applications of Pulsed Electric Field Treatments for the Enhancement of Mass Transfer from Vegetable Tissue. Food Eng. Rev. 2010, 2, 109–130. [CrossRef]

Olmo-García, L.; Bajoub, A.; Benlamaalam, S.; Hurtado-Fernández, E.; Bagur-González, M.; Chigr, M.; Mbarki, M.; Fernández- Gutiérrez, A.; Carrasco-Pancorbo, A. Establishing the phenolic composition of Olea europaea L. leaves from cultivars grown in morocco as a crucial step towards their subsequent exploitation. Molecules 2018, 23, 2524. [CrossRef] [PubMed]

Palmeri, R.; Parafati, L.; Trippa, D.; Siracusa, L.; Arena, E.; Restuccia, C.; Fallico, B. Addition of olive leaf extract (ole) for producing fortified fresh pasteurized milk with an extended shelf life. Antioxidants 2019, 8, 255. [CrossRef] [PubMed]

Brahmi, F.; Mechri, B.; Dabbou, S.; Dhibi, M.; Hammami, M. The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. Ind. Crops Prod. 2012, 38, 146–152. [CrossRef]

Brahmi, F.; Mechri, B.; Dhibi, M.; Hammami, M. Variations in phenolic compounds and antiradical scavenging activity of Olea europaea leaves and fruits extracts collected in two different seasons. Ind. Crops Prod. 2013, 49, 256–264. [CrossRef]

El, S.N.; Karakaya, S. Olive tree (Olea europaea) leaves: Potential beneficial effects on human health. Nutr. Rev. 2009, 67, 632–638. [CrossRef]

Cifa, D.; Skrt, M.; Pittia, P.; Di Mattia, C.; Poklar Ulrih, N. Enhanced yield of oleuropein from olive leaves using ultrasound-assisted extraction. Food Sci. Nutr. 2018, 6, 1128–1137. [CrossRef] [PubMed]

Batra, G.; Gortzi, O.; Lalas, S.I.; Galidi, A.; Alibade, A.; Nanos, G.D. Enhanced antioxidant activity of Capsicum annuum L. and

Moringa oleifera L. extracts after encapsulation in microemulsions. ChemEngineering 2017, 1, 15. [CrossRef]

Gabric´, D.; Barba, F.; Roohinejad, S.; Gharibzahedi, S.M.T.; Radojc˘in, M.; Putnik, P.; Bursac´ Kovac˘evic´, D. Pulsed electric fields as an alternative to thermal processing for preservation of nutritive and physicochemical properties of beverages: A review. J. Food Process. Eng. 2018, 41, e12638. [CrossRef]

Niu, D.; Zeng, X.A.; Ren, E.F.; Xu, F.Y.; Li, J.; Wang, M.S.; Wang, R. Review of the application of pulsed electric fields (PEF) technology for food processing in China. Food Res. Int. 2020, 137, 109715. [CrossRef] [PubMed]

Δημοσίευση V

Extraction of volatile aroma compounds from toasted oak wood using pulsed electric field

George V. Ntourtoglou[1], Foteini Drosou[1], Yang Enoch[1], Evangelia A. Tsapou1 Eleni Bozinou[2] Vassilis Athanasiadis[2] Arhontoula Chatzilazarou[1] Euthalia G. Dourtoglou[1] Stavros I. Lalas[2] Vassilis G. Dourtoglou[1]

ABSTRACT

The effect of pulsed electric field (PEF) on the extraction of volatile compounds from toasted oak wood chips immersed in various aqueous ethanol solutions (5%, 12%, 50%, and 70% vol/vol) was determined by Gas chromatography/mass spectrometry (GC/MS) after ultrasound-assisted extraction. PEF treatment showed the highest impact in the 5% solution, increasing vanillin, syringaldehyde, oak lactone (*cis*- and *trans*-), and furfural by 75%, 371%, 13%, and 50%, respectively. PEF was also tested on *Agiorgitiko* red wine and on malt and wine distillates. In wine distillate, 4-vinylguaiacol was also detected. For red wine, a PEF of 1.2 kV/cm increased the efficiency of extraction of wood aroma compounds from 5% to 200%. Differences in the extracted volatile compound concentrations between the control and PEF- treated samples were also observed in the malt and wine distillates. The sensory evaluation showed that the PEF-treated malt was similar to an aged whiskey, having nuances of toasted oak.

PRACTICAL APPLICATIONS

This work was done as a preliminary investigation for our on-going research into the application of PEF technique for the extraction of aromatic compounds from oak wood, and the acceleration of both wine and malt distillate aging. The findings suggest that PEF could be introduced in wine, brandy, and whiskey industry as a novel technology for the acceleration of the aging process.

INTRODUCTION

The pulsed electric field (PEF) technique is based on the use of high intensity PEFs for the disintegration of the cell membrane (Maged and Amer Eiss, 2012; Ntourtoglou et al., 2020; Ortega-Rivas, 2012; Puertolas et al., 2010). With the induction of external electric field on cells, destabilization of the membrane and perforation occur, resulting in the formation of pores in the lipid bilayer or the protein domains of the cell membrane (McLellan et al., 1991). This phenomenon is also known as electroporation. The main parameters determining the efficacy of PEF are intensity, pulse width, pulse number, and frequency. Depending on the intensity and the duration of the electric field, the formed pores can either reseal (reversible electroporation) or lead to cell disruption (irreversible electroporation) (Ortega-Rivas, 2012; Timmermans et al., 2014). Regarding small molecules, Kandušer and Miklavčič (2008) suggested that short pulses of microseconds to milliseconds are sufficient, whereas for large molecules, like dextranes 70 kDa or DNA, longer pulses in the range of few milliseconds should be used. However, the exact mechanism and the impact of each factor have to be further elucidated.

The high intensity PEF processing system is a simple electrical system consisting of a high-voltage source, a capacitor bank, a switch, and a treatment chamber. From the electric point of view, the PEF treatment chamber represents the electric load consisting of two or more electrodes filled with the liquid substance to be treated. The chamber has to be constructed in such a way that the electric field acting on the liquid is more or less homogeneous across the entire active region. This goal can be reached in principle with planer, coaxial, and axial electrode geometries (Loeffler, 2006).

In the past few years, there has been an increasing interest in processing microorganisms with PEF. The application of PEF is primarily used as a nonthermal process for the inactivation of microorganisms with the potential of being an alternative for the pasteurization of food without downgrading its sensory and nutritional properties (Álvarez et al., 2003; Grahl & Markl, 1996; Ntourtoglou et al., 2020; Toepfl et al., 2007).

Increasing interest also appears in the application of electric field on wine for a number of purposes as well. Corrales et al. (2008) compared the effect of PEF and other novel technologies on the extractability of anthocyanins from grape by-products. The effect of PEF on (+)-catechin–acetaldehyde condensation in a model wine solution was studied by Zhao et al. (2013). Their study showed that the condensation between (+)-catechin and acetaldehyde was apparently enhanced by PEF treatment. Under treatment at 40 kV/cm, the condensation was increased with increasing reaction temperature, as well as with decreasing pH values. The work of Puertolas et al. (2010) showed that each grape variety behaves differently in response to PEF treatment. Taking into consideration that the efficacy of PEF treatments depends on the cell size and the homogeneity of the medium, it could be suggested that the cell morphology among grapes influences the effectiveness of PEF. It was observed that, when PEF treatment is performed prior to vinification, the optimum electric field strength of PEF treatment for the anthocyanin extraction from Cabernet Sauvignon grapes was 5 kV/cm, whereas for Merlot grapes, it was 7 kV/cm. According to another study by Delsart et al. (2014), which is in accordance with the results of Puertolas et al. (2010), when PEF treatment was applied to Cabernet Sauvignon grapes before fermentation, treatments of long duration and low electric field strength (E = 0.7 kV/ cm, t_{PEF} = 200 ms, W = 31 Wh/kg) mainly affected parietal tannins, while high electric field strength and short duration treatment (E = 4 kV/cm, t_{PEF} = 1 ms, W = 4 Wh/kg) led to greater extraction of the anthocyanins.

The influence of PEF on wood material was described by Kumar et al. (2011). After the treatment of wood chips with an electric field of 1–10 kV/cm, they observed an increase in tissue porosity, a phenomenon that may be due to cellulose hydrolysis. Recently, Kiełbasa et al. (2021) reached to the same conclusion after treating dry sawdust from coniferous trees with PEF by placing it between two high conductive stainless-steel electrodes, applying pulses of 25–30 kV/ cm and measuring the heat of combustion of the treated wood. Additionally, Kumar and Sharma (2017) claimed that PEF pretreatment exposes the cellulose present in the biomass by creating the pores in the cell membrane and, thereby, allowing the entry of agents that will break the cellulose into constituent sugars. Nevertheless, the mechanism that unambiguously determines these phenomena has not been described in detail. Also, Vorobiev and Lebovka (2017) described the application of PEF in lignocellulosic material which has as result the disintegration of lignocellulosic mass.

It is widely known that the use of oak in alcoholic beverages and wine plays a significant role in processing and can have a profound effect on the resulting product (wine, whisky, cognac, etc.) affecting the color, flavor, tannin profile, and texture. Oak comes into contact with the product in the form of a barrel during the fermentation or aging period. It can be also introduced in the form of free-floating oak chips or as wood

staves (or sticks). The wood chips are used for the rapid flavoring of alcoholic beverages. Their use greatly speeds up the flavoring or aging compared with a barrel—and specifically, the pieces from American oak (*Quercus alba*) offer intense aroma of vanilla and coconut and more bitterness and astringency. Seven of the main substances extracted from wood during the aging process, form the sensory character. These are two phenolic aldehydes (vanillin and syringaldehyde), two phenolic alcohols (eugenol and guaiacol), furfural, and oak lactone (the *cis*-isomers and *trans*- isomers of β-methyl-octalactone). However, according to Arapitsas et al. (2004) and Rodriguez-Rodriguez and Gomez-Plaza (2012), the most important volatile compounds for flavor processing that are extracted from oak wood are syringaldehyde, vanillin, oak lactone, and furfural.

This work was done as a preliminary investigation for our ongoing research into the application of PEF techniques in the extraction of aromatic compounds from oak wood and the acceleration of both wine and distillate maturation. Specifically, the effect of PEF on the extraction of volatile compounds from toasted wood chips immersed in various aqueous ethanol solutions was determined by Gas chromatography/mass spectrometry (GC/MS) after ultrasound assisted extraction. PEF effect was also studied on Agiorgitiko red wine and on malt and wine distillates.

MATERIALS AND METHODS

Oak chips

Untoasted French *Quercus* spp. oak wood chips were purchased from Arobois (Gagnac, France). These oak wood chips were extra small in size (definition of the size from the producer) (8 mm long and 3 mm thick) and heterogeneous in shape, mainly rhombohedral. The chips were toasted at 200°C for 2 hr before being used. Toast was performed according to Arapitsas et al. (2004).

Chemical and reagents

Absolute ethanol (purity > 99.8%) was purchased from Merck (Darmstadt, Germany). Dichloromethane, chloroform, sodium chloride, and anhydrous sodium sulfate were purchased from Chem Lab (Zedelgem, Belgium). Vanillin, syringaldehyde, oak lactone, and furfural were purchased from Sigma-Aldrich (Darmstadt, Germany).

Aqueous solution of ethanol

The effect of PEF on the extraction of volatile compounds from oak wood chips was investigated in four different alcoholic concentrations (5%, 12%, 50%, and 70% vol/vol). For the preparation of each aqueous solution of ethanol, absolute ethanol (purity > 99.8%) was diluted with distilled water to the desired alcoholic volume. The dielectric constants (ε_1) of the ethanol solutions were calculated as 77.22 for the 5% (vol/vol) solution, 73.32 for the 12% (vol/vol) solution, 52.15 for the 50% (vol/vol) solution, and, finally, 41.01 for the 70% (vol/vol) solution.

Wine

The effect of PEF on the extraction of volatile compounds from oak wood chips was investigated in wine produced at the Department of Wine, Vine and Beverage Sciences (University of West Attica) from grapes of Agiorgitiko, a Greek red wine grape variety.

Malt distillate and wine distillate

Malt distillate (alcoholic degree of 50% vol/vol) was purchased from the local market in order to compare the influence of PEF application between model ethanol solution and alcoholic beverage of similar alcoholic concentration. Distillate was chosen under the criteria of having zero interference with oak. Wine distillate was obtained by the distillation of wine produced by Agiorgitiko grapes, just after its stabilization without remaining in barrel (young wine).

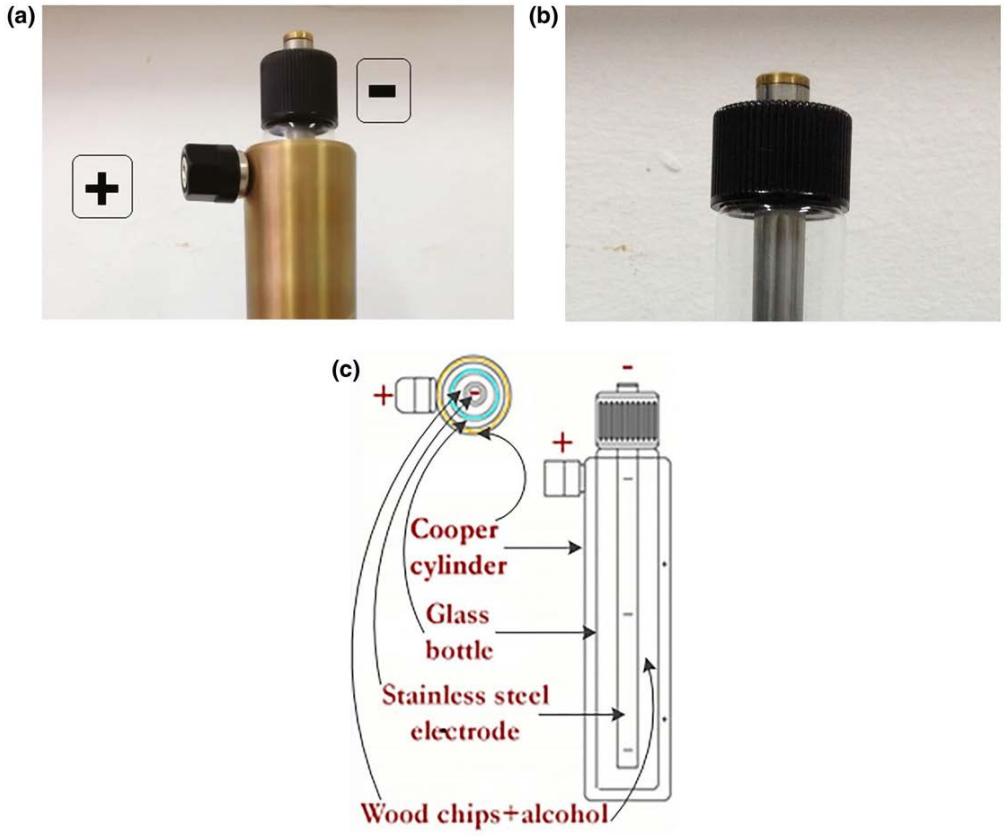


FIGURE 1 The pulsed electric field (PEF) treatment chamber with details

PEF apparatus

The PEF equipment used was a static bench scale system. It was consisted of a high voltage power generator (Eisco, India), a digital oscilloscope (UTD 2062C, ELV Elektronik AG, Germany), and a pulse generator (UPG 100, ELV Elektronik AG).

The PEF generator used provided a maximum voltage of 5 kV. This generator provided pulses of monopolar rectangular shape. Signals of voltage, current, frequency, and pulse waveform were monitored by the digital oscilloscope.

The processing cell (Val Electronic, Greece) is shown in Figure 1a–c. The outer part consisted of a copper cylinder, which serves as a positive electrode (metal wall 4 mm, length 125 mm, diameter and 28 mm). A “U” shaped glass tube with a screw cap (Figure 1a,b) (20 mm in diameter, 130 mm in height, and 2 mm glass thickness) carrying the negative electrode, filled with the solution to be treated, was inserted in the copper cylinder. The negative electrode was a stainless-steel bar (5 mm in diameter and 120 mm in height) which had been attached to the stopper which was screwed into the “U”-shaped glass tube. The strength of the electric field *Es* is calculated as $Es = V/d$,

where E is the applied voltage to the cell and d is the distance between the negative electrode and the copper cylinder (d = 0.75 cm) (Zhang et al.,. ,2012, 2013).

Using the following equations:

$$Es \times s_1 = Eg \times s_2$$

$$E = Es \times 0.75\,\text{cm} + Eg \times 0.2\,\text{cm}$$

where Es = electric field in the ethanol solution, Eg = electric field in the glass, and E = applied voltage of 5 kV/cm; the Es was calculated as 1.02 (5% ethanol), 1.06 (12% ethanol), 1.40 (50% ethanol), and 1.69 (70% ethanol).

PEF treatment procedure

Toasted oak chips (0.2 g) (mean diameter 2–3 mm) were added to 25 ml of each sample (aqueous ethanol solutions, malt or wine distillates) in the “U”-shaped glass tube. For each case, the time (s) of treatment was calculated as:

$$t = t_i + t_p \times P$$

where t_i = pulse width (s), t_p = pause time (s), and P = number of pulses. The treatment conditions used in the experiments were E = 5 kV/ cm, t = 1,500 s or 25 min (t_i = 5 s, t_p = 5 s, p = 150). Finally, the treatment conditions used in the experiments for malt distillate were E = 5 kV/cm and 25 min (t_i = 2 ms, t_p = 5 s, p = 299) duration. As control samples ethanol solutions with 0.2 g of oak wood chips were used. However, during the experimental procedure, no energy was applied (0 kV/cm).

Extraction of volatiles

Immediately after treatment, all samples, either PEF treated or control, were subjected to ultrasound-assisted extraction. During this procedure, 10 ml of saturated NaCl solution and 25 ml of dichloromethane were added to the samples and mixed in a laboratory flask. The flask was then placed in an ultrasonic bath (Transsonic T570/h, Elma, Germany) for 10 min at room temperature. Then, the samples were centrifuged (Hermle Z200A, Milan, Italy) at 3,500 rpm for 5 min for better separation of phases. The supernatant (aqueous phase) was then extracted again using the same volume of solvent in the ultrasonic bath for 10 min. The combined organic layers were then washed with distilled water in a separation funnel, dried over anhydrous sodium sulfate, filtered, and then condensed in a vacuum rotary evaporator (BUCHI, Rotavapor, R-205, Flawil,

Switzerland). Prior to chromatographic analysis, the sample was recovered with 50 µl of chloroform. One microliter of this solution was used for the GC/MS analysis.

Gas chromatography/mass spectrometry analysis

Each sample was subjected to GC/MS analysis, using an Agilent 6,890 series GC System (Agilent Technologies, California, USA), equipped with 5975C VL MSD and a fused silica capillary column, 30 m × 0.32 mm i.d. × 0.25 µm film thickness (HP-5MS, Agilent Technologies). One microliter of sample was injected using a split ratio of 100:1. The injector temperature was set at 250°C, the carrier gas was helium at a flow rate of 1 ml/min, and the oven temperature program was 50°C for 2.5 min, increased to 180°C at 2.5°C/ min, then to 230°C at 2°C/min, then to 250°C at 6°C/min, hold at 250°C for 5 min, then increased to 270°C at 5°C/min, and, finally, holding for 2 min at 270°C. The temperature of the transfer line was set at 280°C. The mass spectrometer operated in the electron ionization (EI) mode at an ionization voltage of 70 eV in the mass range 40–550 amu and a manifold temperature of 270°C. All data were recorded using the ChemStation software.

The identification of vanillin, syringaldehyde, furfural, and oak

lactone was made using their retention times and by comparing their mass spectra with a spectral library of known standard com- pounds. For the quantification of these compounds, external calibration curves were prepared from standards. The quantification of the compounds was made in Full Scan. The GC peak area of each compound was obtained from the ion extraction chromatogram by selecting target ions for each one. For the malt distillate sample, only vanillin and syringaldehyde were determined.

All the above determinations were carried out in triplicate. Data are reported as mean values of these determinations.

Sensory testing

The aroma characteristics of non PEF-treated and PEF-treated malt were evaluated by five panelists highly experienced in sensory analysis.

Statistical analysis

Statistical analysis for the standard deviation (*SD*) of the means and *t* test was carried out with Excel 2013 (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Extraction of volatile compounds from oak

The extractability of all the volatile compounds that were studied showed a behavior which depended on the alcoholic volume of the solution (Figure 2). Among the non-PEF-treated ethanol solutions (taking into consideration that the process time was the same for all samples), an increase in the concentration of vanillin and syringaldehyde extracted was observed as the alcoholic volume of the solution increased, with the 5% (vol/vol) solution having the lowest concentrations and the 70% (vol/vol) solution having the highest concentrations. This observation can be corroborated with the findings of the extraction of polyphenols in the studies of Tao et al. (2014) and Karvela et al. (2008). The above mentioned authors showed that high ethanol concentrations have high extraction capacity and contribute to more rapid extraction of certain polyphenols from oak wood chips. However, oak lactone showed to be affected to a smaller degree while furfural showed no change in its extracted concentration (Figure 2).PEF treatment showed an increase in the extraction of all compounds examined in 5%, 12%, and 50% (vol/vol) ethanol solutions. Specifically, these compounds showed an augmenting rate of extraction as the alcoholic volume increased, apart from the case of furfural, which showed a decrease but it was always higher in the PEF-treated samples (Figure 2). In general, PEF showed the highest efficacy for the molecular extraction of oak components, in 5% (vol/vol) ethanol solutions. Specifically, in the 5% ethanol solution, all compounds showed an increase in the amount extracted in comparison with that of the non-PEF-treated samples. Furfural, oak lactone, vanillin, and syringaldehyde increased by 85%, 12%, 75%, and 371%, respectively, compared with their non- PEF-treated samples. The respective increase of the compounds mentioned above for the 12% (vol/vol) ethanol solution was 58%, 10%, 15%, and 51% (for furfural, oak lactone, vanillin, and syringaldehyde, respectively). In the case of the 50% (vol/vol) solution, it was 2%, 7%, 35%, and 39%. It can be concluded that the effectiveness of PEF in extracting oak compounds is influenced by the dielectric constant of the medium and the polarity and structure of the molecule. An independent one-tailed *t* test for all the compounds separately, in all the solutions except 70%, was performed in order to evaluate the statistical difference of their concentration with and without PEF treatment. The results gave $p = .09$ for vanillin, $p = .14$ for syringaldehyde, $p = .09$ for furfural, and $p = .05$ for oak lactone. It was shown that only for oak lactone there was a significant and perceptible difference at a 5% probability level. One more independent sample one-tailed *t* test was conducted, in order to evaluate the statistically significant impact of PEF on the total analyzed compounds, but the results gave a $p = .22$. Despite the fact of nonstatistical difference

between samples with and without treatment, their high percentage difference is not negated, in order to evaluate PEF treatment in a downstream process.

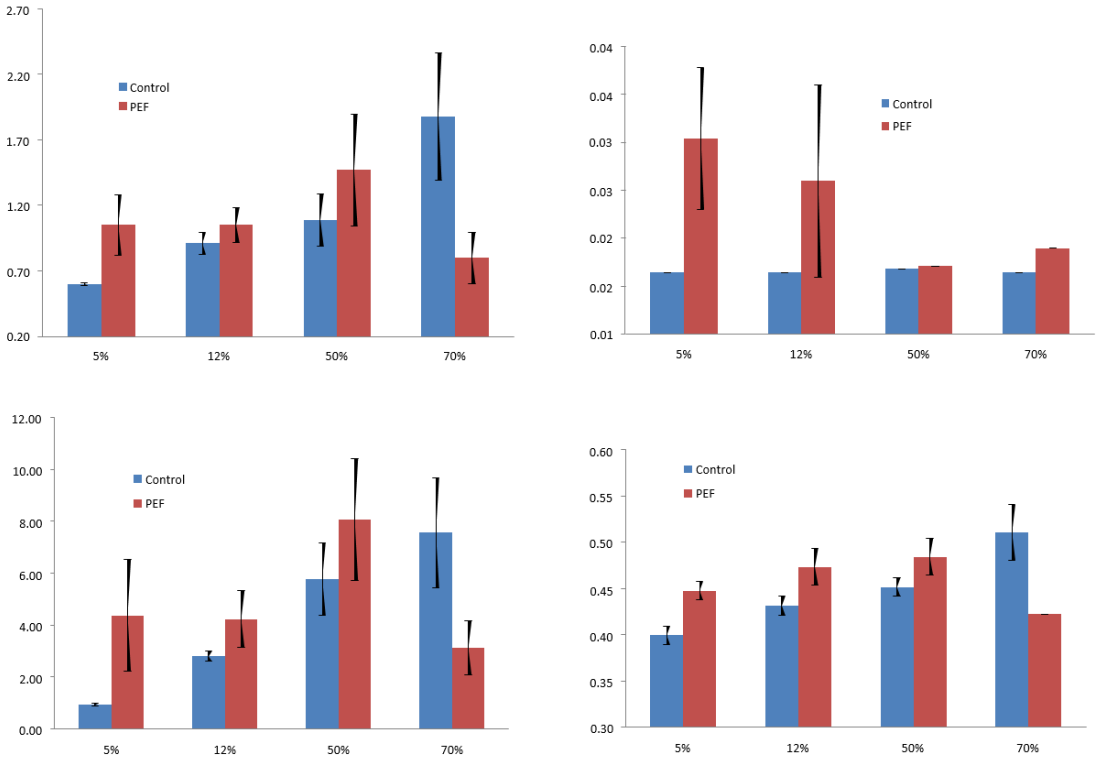


FIGURE 2 Comparative concentrations (mg/L) of untreated and pulsed electric field (PEF)-treated (5 kV/cm) aqueous ethanol solutions (5%, 12%, 50%, and 70% vol/vol) for vanillin, syringaldehyde, oak lactone, and furfural. Data represent the mean values of three replicates with error bars (standard deviation)

On the contrary, a significant difference was observed for vanillin, syringaldehyde, and oak lactone in the 70% (vol/vol) ethanol solutions. Specifically, lower extraction of the compounds was observed compared with their non-PEF-treated solutions. This outcome is coherent to Barbosa-Cánovas and Bermúdez-Aguirre (2010) and Siemer et al. (2014) who stated that the effectiveness of PEF is degraded, when a medium with high conductivity is being treated, due to the demoted electric field being generated across the treatment chamber. Conversely to the above-mentioned statement, furfural showed a 16% increase compared with its non-PEF-treated solution. Among the compounds, regardless of the alcoholic volume, oak lactone seemed to be affected the least when PEF was applied. Therefore, PEF does not seem to contribute in any significant way to the release of oak lactones from oak wood chips.

Volatile compounds of Agiorgitiko red wine

Concerning the PEF treatment of wine, two different voltages and pulse durations were applied in order to find the best combination for the extraction of oak wood compounds. As shown in Figure 3, the lower voltage (1.2 kV/cm) and the long pulse duration (5 s) generated the best results. Specifically, when the treatment conditions were set at E = 1.2 kV/cm and t_i = 5 s, there was an increase in the vanillin, syringaldehyde, furfural, and oak lactone concentration by 60%, 18%, 22%, and 83%, respectively. When an E = 5 kV/ cm and t_i = 0.002 s were used, an increase in the extraction of all volatile compounds also appeared, apart from the case of oak lactone which remained at the same concentration. The other two conditions tested (E = 5 kV/cm and t_i = 5 s and E = 1.2 kV/cm and t_i = 0.002 s) showed an almost equal result on the percentage of extraction of three of four volatiles (apart from the case of vanillin where the most intense conditions extracted 11% more). As indicated previously, the efficacy of PEF treatments depends on the cell size and homogeneity of the medium. Each grape variety behaves differently in response to PEF treatment. In the case of Agiorgitiko red wine, lower voltage and long pulse duration are more appropriate.

Volatile compounds of malt and wine distillates

Differences in the extracted concentrations between the PEF- treated samples were also observed in the malt distillate (treated with E = 5 kV/cm for 25 min [t_i = 5 s]) (Figure 4). In contrast to the control, which presented 0.6 mg/L of vanillin and 0.8 mg/L of syringaldehyde, the PEF-treated malt presented a higher content of these volatiles (28.6% and 101.4%, respectively). A similar phenomenon (concerning polyphenols this time) was observed by Karvela et al. (2008). The sensory evaluation of both control and PEF-treated malt showed that the PEF-treated malt were similar to in barrel- aged whiskey, having nuances of toasted oak. In wine distillate, 4-vinylguaiacol was detected for first time, probably due to different wood composition. In malt distillate, *cis*-oak lactone and *trans*-oak lactone were distinguished, a fact that proves that the effect of PEF was positive to both isomers (Figure 5.

Previous studies using electric field showed similar results to those of the present work. Specifically, Zhang et al. (2012, 2013) used electric field treatment (1 kV/cm applied every 12 hr in 5-L oak barrels) in brandy and showed that the treatment enhanced the extraction of volatile compounds from oak barrels. Additionally, they observed that electric field resulted in a 25% enhancement of vanillin after 150 days of treatment. As for syringaldehyde, the enhancement after 90 days was 16.6% (Zhang et al., 2012).

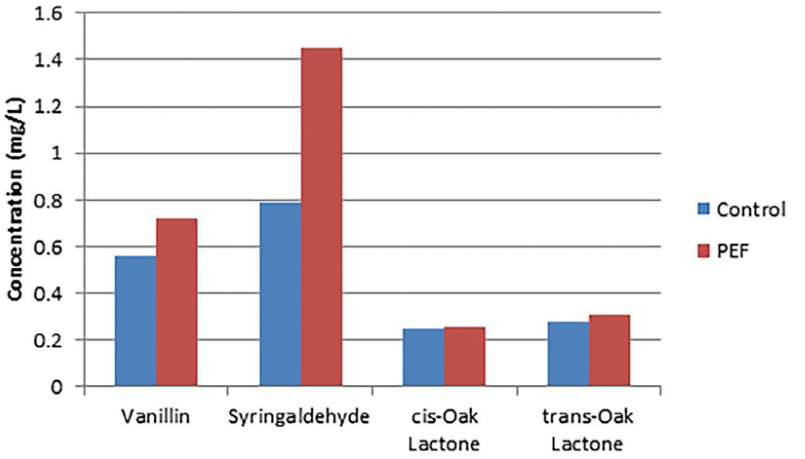


FIGURE 4 Comparative concentrations(mg/L) of control and PEF-treated (5 kV/cm) malt spirit. Data represents the mean values of three replicates

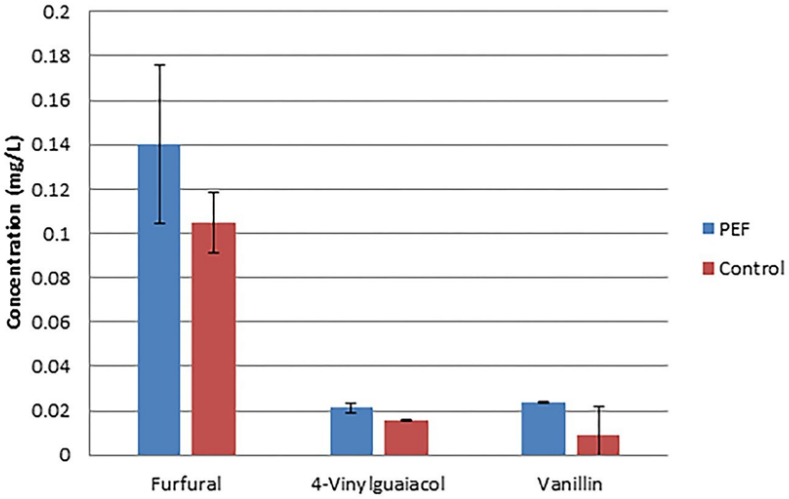


FIGURE 5 Comparative concentrations (mg/L) of untreated and pulsed electric field (PEF)-treated (5 kV/cm) wine spirit. Data represent the mean values of two replicates with error bars (standard deviation)

The PEF cell used during this study was actually a two-dielectric capacitor: one was the alcoholic solution and the other was glass. Additionally, toasted wood chips were a polymer with dielectric properties. Toasting of wood chips is the process of heating wood to produce flavor compounds not present in raw wood. This procedure breaks down the molecular structure of hemicellulose, lignin, and cellulose of wood creating that way small molecules, the aromatic compounds (Jordão et al., 2006; Martínez-Gil et al., 2018), with dielectric properties different from those of wood. These were attached to the wood matrix with different forces (van der Waals or hydrogen intermolecular bonds). When voltage was applied, an orientation of dielectrics (wood chips) in the lines of the electric field took place, having as result the breakdown of intermolecular forces and releasing these molecules into the alcoholic solution. Because the aromatic molecules were dielectrics, they were also oriented in the direction of the field. The medium (alcohol/water), which by its nature was suitable for the extraction of these molecules, was rapidly enriched. When the electric circuit was open, there was no field and the wood chips took a new position inside the field. However, when a new pulse

was (re)applied, again, a reorientation and extraction took place. Therefore, we believe that this phenomenon was definitely the effect of the electric field and because the field was not continuous and was applied by pulse, we claim a PEF effect.

CONCLUSIONS

This work was done as a preliminary investigation for our on-going research into the application of PEF techniques for the extraction of aromatic compounds from oak wood and the acceleration of both wine and malt distillate aging. During this study, the effect of PEF in the extraction of certain volatile compounds from oak in aqueous ethanol solution was determined. All compounds (vanillin, syringaldehyde, furfural, and oak lactone) showed a behavior which depended on the alcoholic volume of the solution when they were exposed to PEF. In the 5%, 12%, and 50% (vol/vol) ethanol solutions, PEF treatment enhanced the extraction of the compounds compared with their respectively nontreated samples. However, in the 70% (v/v) ethanol solution, PEF resulted in a decreased extraction of compounds. Optimum efficacy of PEF was observed in the 5% (vol/vol) solution, but the 12% and 50% (vol/vol) solutions showed also very good results. The PEF treatment was also tested on Agiorgitiko red wine (E = 5 kV/cm [ti = 0.002 s and ti = 5 s] and E = 1.2 kV/cm [t = 0.002 s and t = 5 s] all for 25-min duration] and a malt distillate (E = 5 kV/cm, ti = 5 s). In the case of the red wine, the lower voltage and a long pulse duration had the best results. Differences in the extracted concentrations between the non-PEF- treated samples were observed in the malt distillate. Its sensory evaluation showed that the PEF-treated malt distillate was similar to an aged whiskey, having nuances of toasted oak. Thus, PEF could be introduced in wine and whiskey industry as a novel technology for the acceleration of aging. Therefore, PEF technology could replace the use of oak barrels for the aging of alcoholic beverages and obviate the drawbacks of barrel use, such as inherent costs, labor requirements, and time.

ACKNOWLEDGMENTS

This work was partially cofinanced by the European Union (European Social Fund [ESF]) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF)—Research Funding Program: ARCHIMEDES III—Investing in knowledge society through the European Social Fund.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Formal analysis; Methodology; Validation; Writing-review & editing: George V. Ntourtoglou. Formal analysis; Methodology: Foteini Drosou. Formal analysis; Methodology: Yang Enoch. Formal analysis; Investigation; Methodology: Evangelia A. Tsapou. Methodology; Writing-original draft; Writing-review & editing: Eleni Bozinou. Formal analysis: Vassilis Athanasiadis. Writing-original draft; Writing-review & editing: Arhontoula Chatzilazarou. Conceptualization; Resources; Writing-review & editing: Euthalia G. Dourtoglou. Conceptualization; Resources; Supervision; Writing-review & editing: Vassilis G. Dourtoglou.

DATA AVAIL ABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

REFERENCES

Álvarez, I., Pagán, R., Condón, S., & Raso, J. (2003). The influence of pro- cess parameters for the inactivation of Listeria monocytogenes by pulsed electric fields. International Journal of Food Microbiology, 87, 87–95. https://doi.org/10.1016/S0168-1605(03)00056-4

Arapitsas, P., Antonopoulos, A., Stefanou, E., & Dourtoglou, V. G. (2004). Artificial aging of wines using oak chips. Food Chemistry, 86, 563– 570. https://doi.org/10.1016/j.foodchem.2003.10.003

Barbosa-Cánovas, G. V., & Bermúdez-Aguirre, D. (2010). Novel food pro- cessing technologies and regulatory hurdles. In C. E. Boisrobert, A. Stjepanovic, S. Oh, & H. L. M. Lelieveld (Eds.), Ensuring global food safety (pp. 281–288). Academic Press. https://doi.org/10.1016/ B978-0-12-374845-4.00016-3

Corrales, M., Toepfl, S., Butz, P., Knorr, D., & Tauscher, B. (2008). Extraction of anthocyanins from grape by-products assisted by ul- trasonics, high hydrostatic pressure or pulsed electric fields: A com- parison. Innovative Food Science and Emerging Technologies, 9, 85–91. https://doi.org/10.1016/j.ifset.2007.06.002

Delsart, C., Cholet, C., Ghidossi, R., Grimi, N., Gontier, E., Geny, L., Vorobiev, E., & Mietton-Peuchot, M. (2014). Effects of pulsed elec- tric fields on cabernet sauvignon grape berries and on the character- istics of wines. Food and Bioprocess Technology, 7, 424–436. https:// doi.org/10.1007/s11947-012-1039-7

Grahl, T., & Markl, H. (1996). Killing of microorganisms by pulsed electric fields. Applied Microbiology and Biotechnology, 45, 148–157. https:// doi.org/10.1007/s002530050663

Jordão, A. M., Ricardo-da-Silva, J. M., Laureano, O., Adams, A., Demyttenaere, J., Verhé, R., & De Kimpe, N. (2006). Volatile compo- sition analysis by solid-phase microextraction applied to oak wood used in cooperage (Quercus pyrenaica and Quercus petraea): Effect of botanical species and toasting process. Journal of Wood Science, 52, 514–521. https://doi.org/10.1007/s10086-005-0796-6

Kandušer, M., & Miklavčič, D. (2008). Electroporation in biological cell and tissue: An overview. In E. Vorobiev, & N. Lebovka (Eds.), Electrotechnologies for extraction from food plants and biomaterials (pp. 1–37). Springer. https://doi.org/10.1007/978-0-387-79374-0_1

Karvela, E., Makris, D. P., Kefalas, P., & Moutounet, M. (2008). Extraction of phenolics in liquid model matrices containing oak chips: Kinetics, liquid chromatography-mass spectroscopy characterization and as- sociation in vitro antiradical activity. Food Chemistry, 110, 263–272. https://doi.org/10.1016/j.foodchem.2008.02.001

Kiełbasa, P., Dróżdż, T., & Popardowski, E. (2021). Influence of coniferous wood conditioning by pulsed electric field on its combustion heat characteristics. Applied Sciences, 11, 983. https://doi.org/10.3390/ app11030983

Kumar, A. K., & Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. Bioresources and Bioprocessing, 4, 7. https://doi.org/10.1186/s40643-017-0137-9 Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2011). Pulsed electric field pretreatment of switchgrass and wood chip species for biofuel production. Industrial & Engineering Chemistry Research, 50,

10996–11001. https://doi.org/10.1021/ie200555u

Loeffler, M. J. (2006). Generation and application of high intensity pulsed electric fields. In J. Raso, & V. Heinz (Eds.), Pulsed electric field tech- nology for the food industry: Fundamentals and applications (pp. 27–72). Springer. https://doi.org/10.1007/978-0-387-31122-7_2

Maged, E. A. M., & Amer Eiss, A. H. (2012). In A. Amer Eissa (Ed.), Pulsed electric fields for food processing technology (pp. 275–306). InTech. https://doi.org/10.5772/48678

Martínez-Gil, A., del Alamo-Sanza, M., Sánchez-Gómez, R., & Nevares,

I. (2018). Different Woods in cooperage for oenology: A review.

Beverages, 4, 94. https://doi.org/10.3390/beverages4040094 McLellan, M. R., Kime, R. L., & Lind, L. R. (1991). Electroplasmolysis and other treatments to improve apple juice yield. Journal of the Science of Food and Agriculture, 75, 303–306. https://doi.org/10.1002/jsfa.2740570214

Ntourtoglou, G., Tsapou, E. A., Drosou, F., Bozinou, E., Lalas, S., Tataridis, P., & Dourtoglou, V. (2020). Pulsed electric field extraction of α and β-acids from pellets of Humulus lupulus (Hop). Frontiers in Bioengineering and Biotechnology, 8, 297. https://doi.org/10.3389/ fbioe.2020.00297

Ortega-Rivas, E. (2012). High-voltage pulsed electric fields. In E. Ortega- Rivas (Ed.), Non-thermal food engineering operations (pp. 275–300). Springer. https://doi.org/10.1007/978-1-4614-2038-5_13

Puertolas, E., Lopez, N., Condon, S., Alvarez, I., & Raso, J. (2010). Potential applications of PEF to improve red wine quality. Trends in Food Science & Technology, 21, 247–255. https://doi.org/10.1016/j. tifs.2010.02.002

Rodriguez-Rodriguez, P., & Gomez-Plaza, E. (2012). Dependence of oak- related compounds on the physiochemical characteristics of barrel- aged wines. Food Technology and Biotechnology, 50, 59–65. https:// hrcak.srce.hr/78999

Siemer, C., Toepfl, S., & Heinz, V. (2014). Inactivation of Bacillus sub- tilis spores by pulsed electric fields (PEF) in combination with thermal energy – I. Influence of process- and product parame- ters. Food Control, 39, 163–171. https://doi.org/10.1016/j.foodc ont.2013.10.025

Tao, Y., Zhang, Z., & Sun, D. (2014). Experimental and modeling stud- ies of ultrasound-assisted release of phenolics from oak chips into model wine. Ultrasonics Sonochemistry, 21, 1839–1848. https://doi. org/10.1016/j.ultsonch.2014.03.016

Timmermans, R. A. H., Nierop Groot, M. N., Nederhoff, A. L., van Boekel,

M. A. J. S., Matser, A. M., & Mastwijk, H. C. (2014). Pulsed electric field processing of different fruit juices: Impact of pH and tempera- ture on inactivation of spoilage and pathogenic micro-organisms. International Journal of Food Microbiology, 173, 105–111. https://doi.

org/10.1016/j.ijfoodmicro.2013.12.022

Toepfl, S., Heinz, V., & Knorr, D. (2007). High intensity pulsed elec- tric fields applied for food preservation. Chemical Engineering and Processing: Process Intensification, 46, 537–546. https://doi. org/10.1016/j.cep.2006.07.011

Vorobiev, E., & Lebovka, N. (2017). Application of pulsed electric energy for lignocellulosic biorefinery. In D. Miklavčič (Ed.), Handbook of electroporation (pp. 2843–2861). Springer. https://doi.org/10.1007/978- 3-319-32886-7_157

Zhang, B., Zeng, X., Lin, W. T., Sun, D., & Cai, J. (2013). Effects of electric field treatments on phenol compounds of brandy aging in oak bar- rels. Innovative Food Science and Emerging Technologies, 20, 106–114. https://doi.org/10.1016/j.ifset.2013.07.003

Zhang, B., Zeng, X. A., Sun, D., Yu, S. J., Yang, M. F., & Ma, S. (2012). Effect of electric field treatments on brandy aging in oak barrels. Food and Bioprocess Technology, 6, 1635–1643. https://doi.org/10.1007/s1194

7-012-0788-7

Zhao, D., Zeng, X., Sun, D., & Liu, D. (2013). Effects of pulsed electric field treatment on (+)-catechin–acetaldehyde condensation. Innovative Food Science and Emerging Technologies, 20, 100–105. https://doi. org/10.1016/j.ifset.2013.07.007

Δημοσίευση VI

Pulsed Electric Field and *Salvia officinalis* L. Leaves: A Successful Combination for the Extraction of High Value Added Compounds

Vassilis Athanasiadis[1], Achillia Lakka[1], Dimitrios Palaiogiannis[1], Vasileios M. Pappas[1], Eleni Bozinou[1], George Ntourtoglou[1,2], Dimitris P. Makris[1], Vassilis G. Dourtoglou[2] and Stavros I. Lalas[1,*]

1. Department of Food Science & Nutrition, University of Thessaly, Terma N. Temponera Str., GR-43100 Karditsa, Greece; vaathanasiadis@uth.gr (V.A.); achlakka@uth.gr (A.L.); dipaleog@med.uth.gr (D.P.); vpap@uth.gr (V.M.P.); empozinou@uth.gr (E.B.); gntourtoglou@uniwa.gr (G.N.); dimitrismakris@uth.gr (D.P.M.)
2. Department of Wine, Vine, & Beverage Sciences, School of Food Science, University of West Attica, Ag. Spyridonos Str., GR-12243 Egaleo, Athens, Greece; vdourt@uniwa.gr

ABSTRACT

The present study aimed to evaluate the pulsed electric field (PEF)-assisted extraction of phytochemicals from *Salvia officinalis* L. leaves. The study parameters included a PEF pulse duration of 10 or 100 μs for 30 min, using different “green” extraction solvents: pure ethanol, pure water, and their mixtures at 25, 50, and 75% v/v concentrations. The resulting extracts were evaluated against reference extracts obtained without PEF. For estimation of the extraction efficiency, the content in total polyphenols, individual polyphenols, and volatile compounds, as well as the resistance to oxidation, were determined. The optimal PEF contribution on the total and individual polyphenols, rosmarinic acid, extractability (up to 73.2% and 403.1% increase, respectively) was obtained by 25% v/v aqueous ethanol solvent using a pulse duration of 100 μs. PEF was proven to also affect the final concentration and composition of volatile compounds of the extracts obtained.

Keywords: pulsed electric field; *Salvia officinalis* L. leaves; extraction optimization; polyphenols; green extraction

INTRODUCTION

Salvia officinalis L. (garden sage) is a Mediterranean native perennial, evergreen aromatic subshrub, belonging to the family of Labiatae/Lamiaceae. The plant serves as an aromatic agent (food flavoring, cosmetics industry) and a medicinal plant [1,2]. The flowers, leaves, and stems are the main parts of pharmaceutical importance [1], with the leaves being the most interesting both for the medicine and food industry. *Salvia officinalis* L. leaves (SOLL) contain a vast phytochemical amount [3]. Sharma et al. [4] reported 160 polyphenolic compounds including caffeic, rosmarinic acid, quercetin, and other flavonoids and phenolic acids. Mono-, di- and tri-terpenes (1,8-cineol, carnosic acid, carnosol, or ursolic acid) are also known to be contained in SOLL. The composition varies depending on the locality, seasonality, extraction solvent, and chosen extraction procedure [5].

For the production of SOLL extracts, several techniques have been thoroughly investigated. These techniques include maceration, ultrasonic-assisted extraction, microwave assisted extraction, supercritical fluid extraction, isolation of volatile compounds, and Soxhlet extraction [6–11]. However, the negative environmental impact that accompanies reduced extraction selectivity and the thermal decomposition of sensitive phytochemicals, as well as the high cost and energy demand of the above technologies, reveals the need for greener technologies (more energy-efficient and environmentally friendly) for the achievement of higher process efficiency [12]. Furthermore, the choice of dried instead of fresh leaves has recently met strong opposition due to phytochemicals' thermal decomposition or thermolabile compound oxidative condensation during plant drying [13,14].

Pulsed electric field (PEF) is an emerging eco-extraction technique of biologically active compounds (BACs). It is non-thermal with minimum energy requirements suitable for green solvents. PEF technology complies with environmental requirements for sustainable production systems [15]. The degree of the PEF's efficiency in assisting the extraction of intracellular solutes from fresh plant materials relies on achieving a periodical pore formation to the lipid bilayer of the cell membrane, caused by the high voltage of PEF application. Ideally, by applying minimum specific energy through PEF application, electroporation occurs in such a way that components of interest inside the cell migrate outside the cell (where the solvent carries them away in solution) resulting in a mass transfer increase and, therefore, extraction yield improvement. The set of PEF parameters that require fine-tuning to enhance the extraction degree for a specific solid–liquid system include the strength of the electric field, the shape, width, and frequency of the pulse, and the duration of the application [5].

The most popular applications of PEFs include microorganism inactivation at high specific energy input levels [16,17], plant material pretreatment for further downstream processing at low to moderate specific energy input levels [18–24] or even the direct extraction of plant material [25]. The first authors that attempted to use PEF technology as the primary method for extraction were Brodelius et al. [26]. Even though, nowadays, attention has accumulated in fine-tuning PEF technology as a primary standalone extraction step for plant material BACs' extraction depending on biomass properties, composition, and degree of comminution, there is still limited knowledge and plenty of room for discovery, innovation, and improvement on the plethora of plant materials and compounds of specific interest.

The scope of this work included investigation into the effect of PEFs on the solid–liquid static extraction of fresh SOLL using green solvents of gradual polarity, well-matched with the phenolic compounds' polarity spectrum (pure ethanol (EtOH), pure water (H2O), and their mixtures at 25, 50, and 75% v/v concentrations). The pulse duration varied between two values under the same period and pulse type. The resulting extracts were evaluated against reference extracts obtained without PEF. The evaluation of the extraction efficiency was performed via determination of the content in total polyphenols (Folin–Ciocalteu method), individual phenolics (high-performance liquid chromatography, HPLC), volatile compounds (headspace solid-phase microextraction coupled to gas chromatography–mass spectrometry, HS-SPME/GC-MS) as well as resistance to oxidation (differential scanning calorimetry, DSC).

The novelty of this work lies upon the use of a non-thermal and eco-friendly technology, PEF, as the primary standalone extraction method for the extraction of *Salvia officinalis* L. BACs (including the thermolabile compounds) in green solvents (pure water, pure ethanol, and their mixtures), using fresh plant material instead of dried. The contribution to the scientific area of interest lies in the lack of similar experimental research on *Salvia officinalis* L.

Materials and Methods

Chemicals

The solvents used for chromatography were of HPLC grade. Formic acid (99%) and acetonitrile were obtained from Carlo Erba Co. (Val de Reuil, France). Sodium carbonate anhydrous (99%) and gallic acid monohydrate were from Penta Co. (Prague, Czech Republic), while Luteolin-7-O-glucoside, caffeic acid, and rosmarinic acid were

from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethanol (99.8%) and Folin–Ciocalteu reagent were acquired from Panreac Co. (Barcelona, Spain).

Plant Material, Handling and Sample Preparation

SOLL used in this study came from a single plant variety provided by a local greenhouse in Karditsa Region—Greece (at 39◦21′53″ North and 21◦56′21″ East and altitude of 105 m, according to Google Earth version 9.142.0.1, Google, Inc., Mountain View, CA, USA). The experiments' series took place within five days (from 07 until 11 December 2020). The average temperature ranged between 7 ◦C and 15 ◦C, and the average relative humidity was 75%. The SOLL were delivered to the lab 5 min after their collection, early in the morning of each experimental day, and processed immediately.

After separation from the branches, SOLL were washed with water to discard impurities and then dried at ambient temperature (24 ◦C) using filter paper until no additional moisture was present on the leaves' surface. Before each extraction trial, the leaves were pulverized (about 0.8 mm diameter) in a blender for 2 min, under identical shear input and batch quantities to ensure homogeneity of the pulverization outcome and minimum temperature rise. The latter resulted in high moisture content powders.

The selected solvent was added to the freshly cut SOLL immediately after grinding and the mixture was subsequently poured into the PEF treatment chamber. In all extraction runs, the raw material to solvent ratio was 1:3 (w/v), utilizing 16 g of SOLL and 48 mL of solvent. After 30 min of extraction, the suspensions were separated by decanting from the plant material, which was then discarded. The suspensions/extracts collected were transferred in a suitable Falcon tube and subjected to clarification via centrifugation (at 10,000 g at ambient temperature for 10 min) for immediate analysis. An infrared thermometer (GM300, Benetech, Shenzhen Jumaoyuan Science and Technology Co., Ltd., Shenzhen, China) was used to monitor the temperature of the treatment chamber contents before and after each extraction. The temperature increments due to the treatment never exceeded a ΔT of 1 ◦C.

Dry Matter Determination

Initially, an adequate amount of each sample batch of pulverized leaves were weighed and subsequently dried at 85 ◦C until constant weight using an oven (Binder BD56, Bohemia, NY, USA). The percentage of moisture and volatiles content was calculated as Equation (1)

$$\%\text{Moisture and volatiles content} = \frac{(W_{BD} - W_{AD})}{W_{BD}} \times 100 \quad (1)$$

where WBD is the weight (g) of pulverized leaves before drying and WAD is the weight (g) of pulverized leaves after drying. The moisture and volatiles content of the leaves was about 80% (w/w). The dry matter (g) determination for each sample was calculated as Equation (2)

$$\text{Dry matter} = Ws - (Ws \times \%\text{Moisture and volatiles content}) \quad (2)$$

where WS is the weight (g) of pulverized leaves without drying used as sample.

Pulsed Electric Field (PEF) Apparatus

An apparatus already presented by Pappas et al. [27] was utilized. In brief, a system comprised of a high voltage power generator (maximum voltage up to 25 kV), a 25 MHz function/arbitrary waveform generator, an electronic switch circuit (IGBTs), and a rectangular treatment chamber made of stainless steel with dimensions: 10 cm × 10 cm × 1 cm.

Extraction Parameters

The investigation boundaries incorporated the solvent used for extraction and the time for PEF treatment. In particular, pulse duration was 10 μs or 100 μs while the pulse period constant was 1000 μs, resulting in an energy input of 0.155 kJ kg^{-1} and 1.55 kJ kg−1, or $2.52x10^{-6}$ KWh and $2.52x10^{-5}$ KWh, respectively. Five solvents were used; pure water, pure ethanol, and their mixtures at 25, 50, and 75% v/v concentrations. The criteria for the choice of the solvents were bound to our desire to utilize the minimum possible quantity of the green organic solvent (ethanol) in the aqueous mixture and our hypothesis that PEF technology would be revealed as beneficial for the extraction of BACs from fresh *Salvia officinalis* leaves using such a mixture. Reference samples were prepared in the very same manner but without the use of PEF, for comparison purposes. All extraction runs were performed in triplicates.

The electrical conductivity of solvents, the strength of the field, the treatment time, and the energy contribution (kJ x kg^{-1}) determinations were measured as we have previously described [27]. Both PEF and reference samples were extracted for 30 min.

Determination of Total Polyphenol Content

The Folin–Ciocalteu assay was carried out as we have previously described [28]. Each sample was diluted to 1:50 (v/v) with deionized water. Next, 0.1 mL of each diluted sample was mixed with 0.1 mL Folin–Ciocalteu reagent into a 1.5 mL Eppendorf tube and was allowed to react for 2 min before the addition of 0.8 mL sodium carbonate (5% w/v). After 20 min of incubation in a water bath at 40 ◦C, the absorbance was obtained at 740 nm. The total polyphenol yield (YTP) was determined as mg of gallic acid

equivalents/g of dry weight (dw) (mg GAE g−1 dw) and based on a gallic acid calibration curve (10–80 mg L−1). A Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany) was used for the determinations.

HPLC

The method was adapted from Kaltsa et al. [29]. In brief, a Shimadzu liquid Chromatograph (CBM-20A) and a Shimadzu detector (SPD-M20A) were used. A Phenomenex Luna C18(2) (100 Å, 5 µm, 4.6 250 mm) (Phenomenex, Inc., Torrance, CA, USA) retained at 40 ◦C, a flow rate was 1 mL min−1, and an injection volume 20 µL were used. The mobile phases and the elution program used have been described previously [29]. Quan- tification calibration curves were prepared using three points (0, 10, and 50 mg mL−1), for caffeic acid (quantified at 320 nm, y = 0.000009x + 0.8755, R2 = 0.9986), rosmarinic acid (at 320 nm, y = 0.00002x + 0.3334, R2 = 0.9998), and luteolin-7-O-glucoside (at 345 nm, y = 0.00002x + 1.0794, R2 = 0.9980). The estimation of the total area was carried out at 245 nm and 350 nm.

Differential Scanning Calorimetry (DSC)

The DSC method used was adapted from Pappas et al. [27]. A Perkin Elmer Diamond DSC (PerkinElmer Inc, Shelton, CT, USA) was used. The antioxidant activity was determined using oxygen as the purge gas. The temperature program was as follows: hold at 40 ◦C for 1 min, heat from 40 to 200 ◦C (40 ◦C/min), and then heat from 20 to 580 ◦C (20 ◦C/min). The starting temperature of oxidation is the onset temperature of the oxidation peak (Tmax).

Volatile Compounds Analysis

The technique (HS-SPME/GC-MS) used was a modification of the method described by Hjelmeland et al. [30]. An SPME fiber coated with a layer of divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA), preconditioned for 30 min at 270 ◦C, was used. For HS-SPME extraction, 10 mL of the sample extract was placed in a 100 mL glass vial; 3 g of NaCl was added and sealed. The vial was kept at 40 ◦C during a 60 min period (10 min for equilibration + 50 min for extraction).

The analysis with GC-MS was carried out according to a modified method described by Hjelmeland et al. [30]. An Agilent Technologies (California, USA) Gas Chromatograph model 7890A equipped with a mass detector (5975C), and a capillary column Agilent J&W DB-1 (30 m 320 µm 0.25 µm) (California, CA, USA) were used. Helium was

used as carrier gas at a flow rate of 1.5 mL min−1. The injector was operated in splitless mode at 240 ◦C. The temperature program was: 40 ◦C for 5 min, increased to 140 ◦C by 2 ◦C/min, and, finally, heated to 240 ◦C by 10 ◦C/min. Volatile compounds were

identified by comparing their mass spectra with data from the integrated NIST 11 library (National Institute of Standards and Technology, Gaithersburg, MD, USA). The peaks were assigned when the similarity was above 80% and the component percentages were calculated as mean values from duplicate GC-MS analysis.

Statistical Analysis

All extraction series and spectrophotometric measurements were executed in triplicate. Microsoft Excel 2019 (Redmond, WA, USA) software was used for the statistical analysis of the results. ANOVA was used for the determination of the statistical significance (at $p < 0.05$) between mean values.

RESULTS AND DISCUSSION

PEF applies millisecond or even microsecond long pulses of various voltages depending on the purpose of its application [31]. It was found that the higher increase of mass transfer rate was achieved by applying a PEF of 0.7–3.0 kV cm−1 and energy input of 1.0–20.0 kJ kg−1. During this study, we attempted to develop a PEF-assisted SOLL polyphenol extraction method under the scope of a green and sustainable standalone process. Moderate electric field intensity of 1 kV cm−1 and short pulses of 10 and 100 μs in a total processing time of 30 min were applied, while mixtures of EtOH–H2O in five different ratios were tested as extraction solvents. In particular, the gradual addition of ethanol to water (25% step gradient) was evaluated to determine whether the PEF effect could offset the increased recovery usually achieved using polar organic solvents in relation to water. EtOH and other polar organic solvents possess a good solubility and, thus, extractability for bioactive phytochemicals, but they must be removed after the end of the treatment, increasing the cost of the whole process. A possible reduction in the need for EtOH when using PEF can result in financial and environmental benefits. To the best of our knowledge, reported studies of SOLL extraction using PEF technology and aqueous organic solvents do not exist in the literature.

Total Phenol Content

According to the results, the highest percentage increase in total phenol (YTP) between PEF and reference extracts was shown by the 25% EtOH solvent, after PEF with 100 μs pulse duration. The 75% EtOH and 100% EtOH solvents also showed significant increases, while pure water and 50% EtOH led to lower increases.

In detail, regarding the pulse duration of 10 μs (Figure 1), the highest percentage increase in YTP between PEF and the reference sample obtained with the 25% EtOH solvent, was 59.00% (significant at $p < 0.05$). Specifically, YTP for the PEF sample was 21.76 mg GAE g−1 dw, while for the reference sample it was 13.68 GAE g−1 dw. The 75% EtOH and 100% EtOH solvents showed significant ($p < 0.05$) increases of 40.48% and 42.18%, respectively. The lowest increases presented with pure water and 50% EtOH were 15.65% and 14.00%, respectively. In these cases, PEF treated and reference samples showed no significant differences.

The results of PEF treatment with a pulse duration of 100 μs are presented in Figure 2. The highest (significant at $p < 0.05$) percentage increase between PEF and the reference sample was achieved again with the 25% EtOH solvent (73.23%), and it was much higher (~24%) compared to that of the pulse duration of 10 μs. In addition, there were slightly higher increases concerning pure water and 50% EtOH (14.40% and 16.97%, respectively). Although the highest percentage increases in YTP between PEF and reference extracts,

for both pulse durations, were achieved with the addition of only 25% EtOH, the highest extraction rate was observed for the PEF-treated samples using 75% EtOH as solvent (Figures 1 and 2). It seems the use of the PEF replenished part of the losses in the extraction rate when a solvent with low concentration of EtOH was used. Additionally, it was shown that longer-duration pulses appeared to deliver higher efficiency regarding the content in SOLL total polyphenols.

Differential Scanning Calorimetry (DSC)

As indicated by Batra et al. [32], this technique can determine the changes in the different physicochemical properties of compounds, which are shown by changes in the heat flow, and therefore, the oxidative stability of a sample can be evaluated through this technique [27]. DSC is the most widely used analytical technique for studies of physical characteristics and thermo-oxidative degradation of fats and oils, as well as their mixtures with herbal plant extracts, according to Kozłowska and Gruczyn´ska [33].

According to the results (Table 1), the maximum peak oxidation (Tmax) was 487 ◦C.The samples that produced this result (the highest antioxidant activity) were those with 75%

EtOH and 10 μs pulse duration. This result was expected since the extracts in 75%

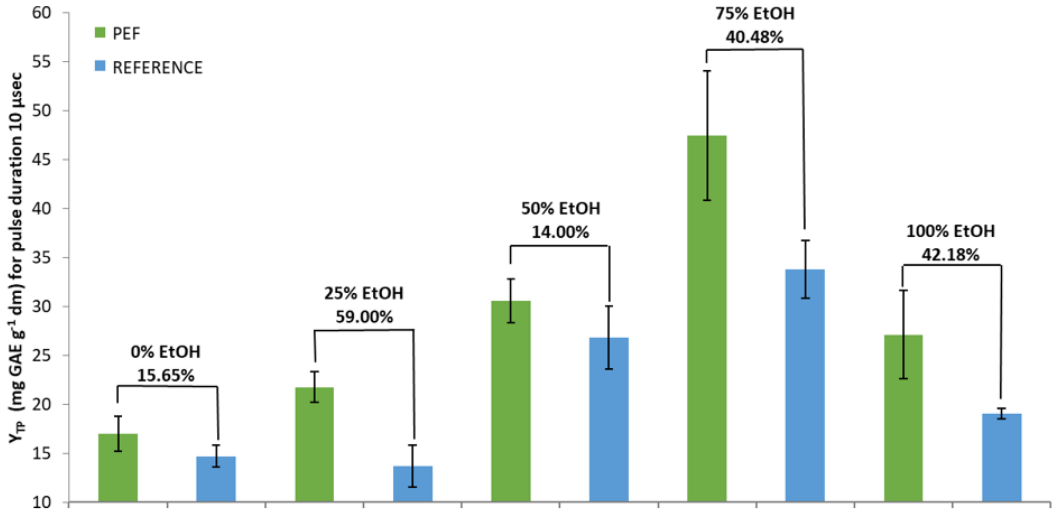


Figure 1. Y_{TP} for PEF-treated and reference extracts in five different tested solvents and a pulse duration of 10 μs. Y_{TP} is the total polyphenol yield.

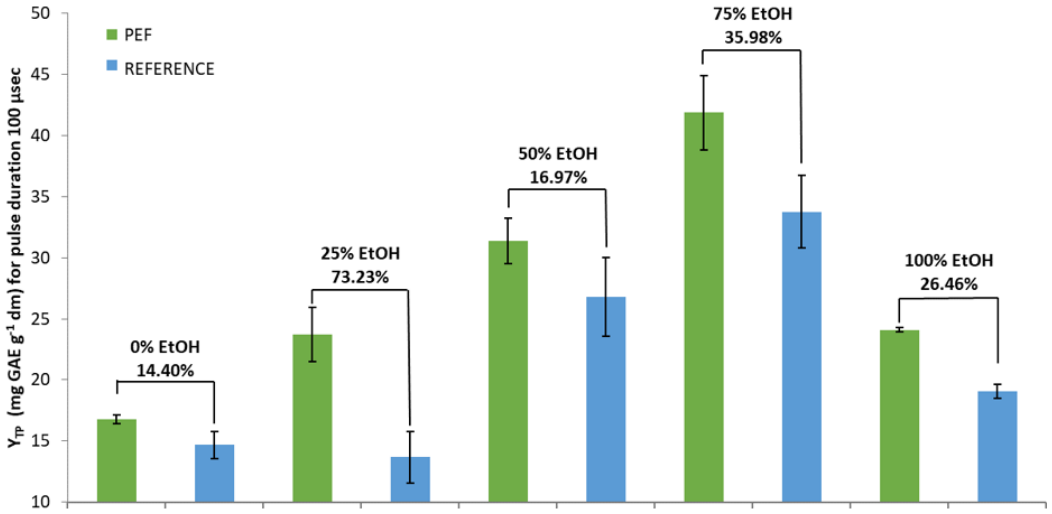


Figure 2. Y_{TP} for PEF treated and Reference extracts in five different tested solvents and a pulse duration of 100 μs. Y_{TP} is the total polyphenol yield.

ethanol were found to have the highest content in total polyphenols. The highest difference in the resistance to oxidation between PEF and reference extracts, expressed by the increase in oxidation temperature (significant at $p < 0.05$), was presented in the extracts produced with 25% EtOH. PEF extracts treated with 100 μs (25% EtOH) reached an average increase in oxidation temperature of 61.5% (in relation to reference extracts), while PEF extracts treated with a pulse duration of 10 μs reached a corresponding increase of 53.8%.

Table 1. Oxidation temperature (T_{max}) of the various samples during DSC determination.

Extraction Solvent Synthesis	PEF Pulse Duration (μs)	T_{max} (°C) of PEF Treated Extract	T_{max} (°C) of Reference Extract	Increase (%)
0% EtOH	10	237 ± 3 [1]	203 ± 2	16.7
	100	221 ± 2		8.9
25% EtOH	10	280 ± 3	182 ± 3	53.8
	100	294 ± 3		61.5
50% EtOH	10	350 ± 2	312 ± 5	12.2
	100	362 ± 4		16.0
75% EtOH	10	487 ± 7	387 ± 8	25.8
	100	462 ± 8		19.4
100% EtOH	10	294 ± 6	257 ± 7	14.4
	100	310 ± 7		20.6

[1] Values are means of triplicate determinations ± Standard deviation.

Volatile Compounds (VCs) Analysis

The results are shown in Table 2. The analysis was carried out only for the samples of 25% EtOH treated with PEF at 100 μs, which displayed the highest percentage increase in YTP and oxidation temperature between PEF and the reference extracts. The peak area obtained by HS-SPME/GC-MS was used to semi-quantify the concentration of different VCs.

Due to the large number of synonyms available, matching compounds by name tags was difficult, as was an accurate and thorough search using chemical IDs (e.g., CAS numbers). The identify compounds show strong MS similarity with library entries, without further information available.

The main components of SOLL previously identified were α- and β-thujone [34], α- and β-pinene, camphor and α-humulene [35], and 1,8-cineole, β-caryophyllene, camphene, myrcene, γ-terpinene, and p-cymene [36,37]. In our study, the principal VCs identified were eucalyptol, β-thujone, D- and L-camphor, 2-bornanol, borneol (endo-borneol), and L-borneol. These main compounds were identified in PEF-treated and reference extracts at about the same total percentage (65.51% and 67.58%, respectively). However, important differences appeared in the percentage of each of the above VCs between the differently treated samples. Specifically, D-camphor, borneol, and L-borneol showed an increase in PEF-treated extract, while eucalyptol, β-thujone, L-camphor, and 2-bornanol decreased. Differences also appeared in many other compounds (Table 2). Additionally, some compounds (p-cymen-8-ol, ( )-trans-pinocarveol, piperitenone, α-santalene, calarene, (+)-epizonarene, (+)-longifolene and ( )-γ-cadinene) appeared only in the PEF treated extract. The compounds p-cymen-8-ol and ( )-γ-cadinene were previously identified in S. officinalis L. leaves extracted by

supercritical CO2 [38] or distillation–extraction [37]. Calarene and ( )-trans-pinocarveol were also previously reported [39,40].

The above results indicate that PEF effects can influence the aroma of SOLL extracts. Another study in line with the above conclusion is that of Sotelo et al. [41]. These authors studied the result of PEF technique on the flavor profile of red-fleshed sweet cherries and concluded that PEF-treated extracts produced higher amounts of volatile compounds that characterize the flavor, and that no adverse compounds appeared because low energy intensities were applied.

Table 2. Percentage of volatile compounds determined in *Salvia officinalis* L. extracts (in 25% EtOH) by HS-SPME/GC-MS.

Compound	RT [1] (min)	Reference	PEF Treated (100 µs)	Compound	RT (min)	Reference	PEF Treated (100 µs)
trans, trans-2,4-Hexadienal	7.798	0.17	0.33	Piperitenone	34.651	nd	0.09
Benzaldehyde	9.907	0.05	Nd [2]	Eugenol	36.322	0.02	0.06
Sabinene	12.190	0.03	nd	3-Carene	36.648	0.02	nd
1-Octen-3-ol	12.548	0.31	nd	α-Cubebene	37.305	0.12	0.10
β-Pinene	13.570	0.18	nd	L-Borneol acetate	38.244	0.04	nd
α-Terpinene	14.918	0.04	0.16	β-Cubebene	39.580	0.04	0.03
p-Cymene	15.099	0.16	0.30	α-Cubebene	39.980	0.01	nd
Eucalyptol	15.777	10.67 [3]	3.35 [3]	α-Gurjunene	40.753	0.09	nd
γ-Terpinene	17.686	0.30	0.23	Caryophyllene	41.089	0.56	0.06
trans-4-Thujanol	18.130	0.41	0.31	Aromandendrene	42.257	0.07	nd
p-Cymenene	19.233	0.07	0.06	δ-Cadinene	42.895	0.03	0.06
2-Carene	19.621	0.11	nd	Humulene	43.034	0.11	nd
α-Thujone	20.110	3.02	1.44	β-Copaene	43.577	0.04	0.04
β-Thujone	21.139	11.07	4.10	δ-Cadinene	44.452	0.02	nd
D-Camphor	22.397	16.05	19.82	Germacrene D	44.641	0.15	0.21
DL-Camphor	22.437	1.38	nd	γ-Cadinene	44.952	0.08	0.07
L-Camphor	22.611	8.23	nd	α-Cadinene	45.147	0.07	0.17
Camphene	22.689	0.47	nd	α-Elemene	45.574	0.07	nd
L-Camphene	23.100	0.71	nd	Epizonarene	45.669	0.08	0.13
D-Pinocamphone	23.388	0.07	nd	α-Muurolene	46.088	0.10	0.18
Isoborneol	23.666	0.17	nd	γ-Muurolene	46.665	0.19	0.22
2-Bornanol	24.350	7.00	2.33	*cis*-Calamenene	46.822	0.07	0.06
Borneol	24.522	3.91	9.56	γ-Elemene	47.050	0.74	1.26
L-Borneol	24.898	10.65	26.35	α-Santalene	47.216	nd	0.81
Borneol	24.970	3.43	nd	δ-Cadinene	47.413	0.30	0.41
p-Cymen-8-ol [4]	25.325	nd	0.17	1,4-Cadinadiene	47.774	0.03	nd
Terpinen-4-ol	25.426	1.21	1.55	α-Calacorene	47.890	0.03	nd
4-Carene	25.787	0.15	0.20	(-)-α-Cadinene	48.106	0.02	0.06
α-Terpineol	26.177	0.57	1.44	Espatulenol	49.771	0.07	0.12
Myrtenol	26.569	0.20	0.52	Caryophyllene oxide	49.928	0.18	0.11
(-)-*trans*-Pinocarveol	26.669	nd	0.10	Diethyl phthalate	50.049	0.10	0.08
cis-Carveol	27.927	0.05	0.10	(+)-γ-Gurjunene	50.714	0.38	0.36
cis-3-Hexenyl valerate	29.787	0.07	nd	α-Guaiene	51.309	0.07	nd
trans-2-Hexenyl valerate	30.328	0.05	nd	Calarene	53.430	nd	0.19
α-Ocimene	30.871	0.07	0.22	(+)-Epizonarene	53.683	nd	0.04
6-Oxocamphor	31.162	0.17	0.55	β-Guaiene	53.714	0.05	nd
Bornyl acetate	32.491	1.65	0.43	Alloaromadendrene	54.477	0.04	nd
Sabinyl acetate	32.980	0.21	0.07	(+)-Longifolene	54.716	nd	0.04
Thymol	33.322	0.11	0.23	(-)-γ-Cadinene	56.080	nd	0.51
Carvacrol	33.789	0.42	0.39	(-)-α-Amorphene	56.134	0.41	nd
Reference extract Total = 87.97%				PEF treated extract Total = 79.80%			

[1] RT: Retention Time; [2] nd: (m/z) spectra were not detected; [3] Blue and green colors denote the highest concentration of compounds; [4] Red color denotes compounds identified only in PEF treated samples.

Extracts' Characterization by HPLC

Evaluation of PEF Effects Based on Extracts' Total Area

Following the results regarding the estimation of YTP and oxidation temperature, the maximum percentage increase in total area between the PEF and reference extracts was reached by using 25% EtOH for both pulse durations. In particular, for pulse duration 10 μs, the increase was 72.83%, while for 100 μs it was 78.72%, both significant at $p < 0.05$ (see Figures S1 and S2, respectively, in Supplementary Materials). For pure water, there were minor changes (not significant—$p > 0.05$). In the case of 50% EtOH, significant increases ($p < 0.05$) of 19.42% for 10 μs and 13.44% for 100 μs appeared. Further addition of EtOH (75%) increased the percentage difference, in the case of the 10 μs pulse duration reaching a greater increase (significant at $p < 0.05$) in total areas than that of 100 μs (72.72% versus 25.46%, respectively). Finally, at 100% EtOH (pure EtOH), a significant increase in PEF- treated samples took place. The percentages were 52.73% versus 36.33% for 10 μs and 100 μs, respectively. It is worth mentioning at this point that there seems to be a clear trend indicating a gradual rise in the extraction yield when increasing the ethanol solvent content from 0% up to 75% EtOH, after which a drop takes place resulting in lower yields when pure ethanol was used.

Polyphenolic Composition

For the estimation of PEF effects in the polyphenolic profile of SOLL, extracts in the optimum EtOH–H2O ratio (25% EtOH) were selected. The main compounds of SOLL found in this work are in line with previous findings [42,43]. As can be seen from the chromatogram of 25% EtOH and 100 μs PEF extract at 320 nm (Figure S3 in Supplementary Materials), four main compounds were identified that belong to the group of phenyl- propanoids and flavones' derivatives. Peak 1 was identified as caffeic acid and peak 4 as rosmarinic acid. The identification was based on the retention time (Figure S3 in Supple- mentary Materials) and absorption spectrum of the compounds and the corresponding reference substances. From our previous works, peaks 2 and 3 were identified as 6-hydroxy luteolin 7-O-glucoside [29] and luteolin 7-O-glucuronide [44], respectively. The amounts of the identified compounds, achieved in the extracts of reference samples processed with 25% EtOH, were 0.76 mg g−1 dw for 6-hydroxy luteolin 7-O-glucoside, 1.04 mg g−1 dw for luteolin 7-O-glucuronide, 0.17 mg g−1 dw for caffeic acid, and 0.37 mg g−1 dw for rosmarinic acid.

Quantification of the main identified components of SOLL was carried out in PEF extracts processed with the optimum EtOH–H2O ratio (25% EtOH) for both 100 μs and 10 μs pulse duration. The corresponding results prove the influence of PEF treatment (Table 3, Figure 3). As indicated by the estimation of YTP, HPLC total area and oxidation temperature, the best performance in the extraction of identified phenolics was achieved by the pulses of 100 μs.

Table 3. Major compounds (mg g^{-1} dw) of *Salvia officinalis* L. PEF treated (pulse duration of 100 μs) and reference extracts, all prepared with 25% ethanol.

PEF Pulse Duration	Compound	PEF Treated Extract	Reference Extract	Increase (%)
100 μs	Caffeic acid	0.24 ± 0.04 [1]	0.17 ± 0.04	41.76
	6-Hydroxy luteolin 7-*O*-glucoside [2]	1.14 ± 0.28	0.76 ± 0.28	49.78
	Luteolin 7-*O*-glucuronide [2]	2.01 ± 0.32	1.04 ± 0.24	93.49
	Rosmarinic acid	1.85 ± 0.82	0.37 ± 0.35	403.12
10 μs	Caffeic acid	0.25 ± 0.04	0.17 ± 0.04	47.37
	6-Hydroxy luteolin 7-*O*-glucoside [2]	0.85 ± 0.07	0.76 ± 0.28	11.49
	Luteolin 7-*O*-glucuronide [2]	1.75 ± 0.35	1.04 ± 0.24	68.01
	Rosmarinic acid	1.67 ± 1.01	0.37 ± 0.35	354.61

[1] Values are means of triplicate determinations ± Standard deviation; [2] 6-hydroxy luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide were quantified as Luteolin 7-*O*-glucoside.

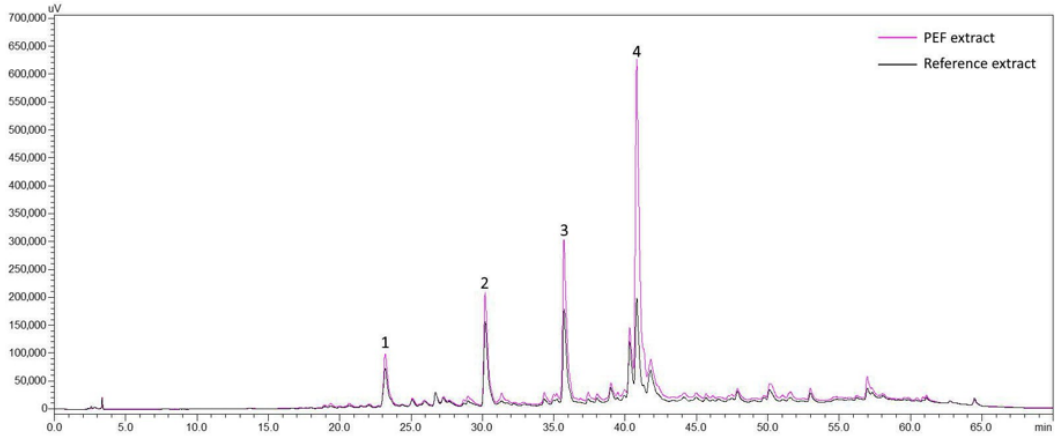


Figure 3. Overlay of chromatograms of PEF-treated and reference extracts at 320 nm with pulse duration 100 μs and extraction solvent 25% EtOH. Peak 1: Caffeic acid; Peak 2: 6-Hydroxy-luteolin-7-*O*-glucoside; Peak 3: Luteolin 7-*O*-glucuronide; Peak 4: Rosmarinic acid. Reference extract obtained without the application of PEF.

Specifically, extraction was enhanced for all examined compounds rising to a 403.12% increase for the 100 μs pulses and up to 354.61% for the 10 μs pulses. This remarkable and significant ($p < 0.05$) increase was shown for rosmarinic acid, achieving 1.85 mg g−1 dw in the optimum sample/extract (25% EtOH, 100 μs). It is well known that beyond the solvents and the extraction method chosen, the seasonality and the locality of a plant rule also the type and the levels of the components detected in plant extracts [5]. However, it is worth mentioning at this point that the above quantity of rosmarinic acid (1.85 mg g−1 dw achieved in the optimum extract) is significantly higher than the amount (0.45 mg g−1 dw) achieved by Bljajic et al. [45] using water ultrasound extraction for 30 min at high temperature (80 ◦C) and a higher liquid to solid ratio (10:1 mL g−1). For caffeic acid and 6-hydroxy- luteolin-7-O-glucoside, the significant ($p < 0.05$) increases for 100 μs pulses, reached 41.76% and 49.78%, respectively. An almost double increase, 93.49% (significant at $p < 0.05$), was reached for luteolin 7-O-glucuronide. It is obvious that both pulse durations succeeded in a permeabilization effect on the membranes and affected the extraction percentage of intracellular

compounds from the cells. The big variance in percentage increases between the main compounds, for the same extraction conditions (Table 3, Figure 4), indicates the potential selectivity of this extraction method, a very important fact considering that the achievement of selective extraction is usually a tedious, time and energy consuming procedure. The main factors that possibly assist this selectivity are the molecular size and structure, the differences in cell membrane disintegration (such as pore size), the solubility of the extracted components, and the solvent's polarity [31,46]. The differentiation of PEF processing parameters, as supported by the literature, also seem to support extraction selectivity. For 6-hydroxy luteolin 7-O-glucoside, in our study, the change in the pulse duration from 10 to 100 μs was followed by a higher increase in the levels of the metabolite, in comparison to the increase obtained for the three other identified metabolites (Table 3, Figure 4). A similar effect was observed during another study performed by our team regarding enhanced polyphenol extraction from olive leaves using PEF [27]. As in the current study, the effect of different duration pulses, 10 and 100 μs, was tested. The amounts of obtained metabolites varied depending on the applied pulse duration. Pulses of 100 μs favored oleuropein recovery, while 10 μs pulses favored the recovery of phenolic glucosides. Thus, the application of different PEF conditions (such as pulse duration of 10 or 100 μs) changes the extraction rate of each molecule promoting the selective extraction of various constituents from the SOLL.

In our work, PEF proved to be a green and effective extraction method. Although the comparison with other techniques cannot be direct because, as mentioned above, the levels of metabolites in each plant depend on both the seasonality and the origin of the plant, the proposed extraction technique can be characterized as efficient, since basic metabolites, identified in this particular study, appear to have been satisfactorily recovered [45]. The developed method can also be considered a green extraction technique because the optimal recovery of metabolites (between PEF- and not PEF-treated samples) was achieved in a low liquid-to-solid ratio (easier solvent removal), with only 25% addition of non-toxic organic solvent, low energy supply, and ambient temperature.

The results showed that PEF boosted the performance of SOLL extraction, revealing new targets for further improvement and insight into this technique. Further work, including process optimization (fine-tuning of important PEF parameters (i.e., number of pulses, etc.), is strongly advisable towards maximizing polyphenol concentration and extraction selectivity. The analysis of the role of solvent polarity in conjunction with PEF should be also evaluated.

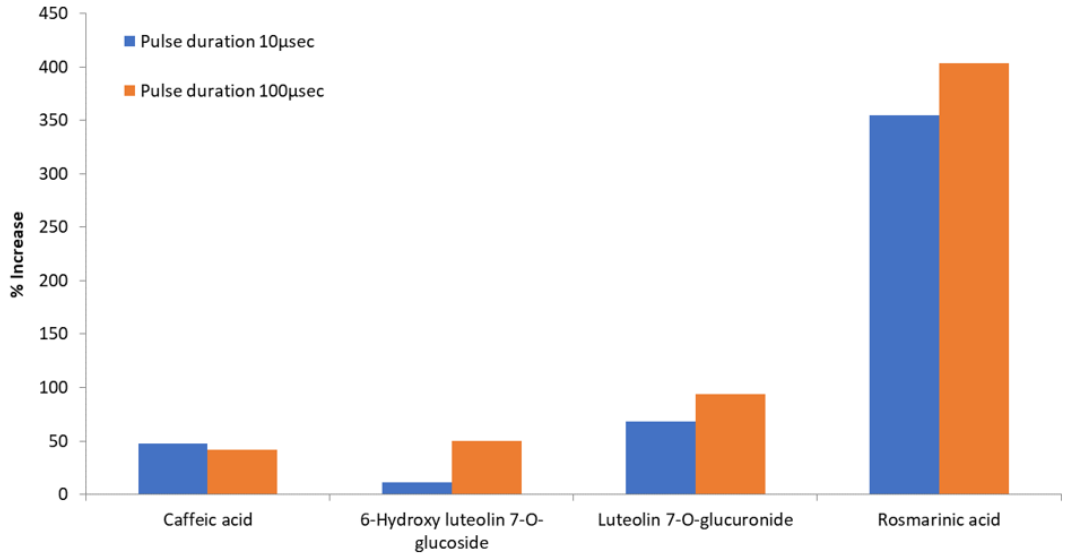


Figure 4. Comparison of the effect (% increase) of different pulse time durations on the extraction of individual phenolics in the same ethanol/water ratio (25% EtOH).

CONCLUSIONS

This work is one of the very few studies that deal with PEF technique (a non-thermal and eco-friendly technology) as the primary standalone extraction step for freshly cut plant material BACs (including the thermolabile compounds) in green solvents. Even though BACs' extraction depends on biomass properties, availability, composition, and degree of comminution, our findings for the specific plant material (*Salvia officinalis* L.) and solvent choice (ethanol, water, and their mixtures) revealed a substantial rise in the polyphenol concentration of the obtained extracts using different PEF conditions. The optimal detected PEF contribution, on the total polyphenol extractability (73.23% increase) and constituents of interest (up to 403.12% increase for specific metabolites) was presented by the 25% v/v aqueous ethanol solvent choice using a pulse of 100 μs for a 30 min extraction duration. The results were verified by the differential scanning calorimetry method, confirming our research target and initial hypothesis of achieving increased levels of extraction rate for the adding value components of the specific fresh plant material utilizing an aqueous green organic solvent with the minimum possible organic solvent content. PEF was proven to affect the final concentration and the composition of VCs in the extracts.

Despite the static nature of the specific extraction technique used in this study, which could be problematic for industrial applications (industries are favored by continuous production procedures), the above results denote that the PEF technique offers excellent potential for green selective extraction of biofunctional compounds from *Salvia officinalis* L. leaves. These compounds can serve in the preparation of high-quality functional foods or cosmetics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10092014/s1, Figure S1: HPLC total area for PEF and Reference extracts in five different tested solvents and a pulse duration of 10 μs, Figure S2: HPLC total area for PEF and Reference extracts in five different tested solvents and a pulse duration of 100 μs, Figure S3: Overlay of chromatograms of extract and reference compounds at 320 nm after PEF with pulse duration 100 μs and extraction solvent 25% EtOH. Peak 1: Caffeic acid; Peak 2: 6-Hydroxy-luteolin-7-O-glucoside; Peak 3: Luteolin 7-O-glucuronide; Peak 4: Rosmarinic acid.

Author Contributions: A.L.: data collection, data interpretation, substantial manuscript prepara- tion. D.P.: data collection, substantial data interpretation, draft manuscript preparation. V.A.: data collection, data interpretation, draft manuscript preparation. V.M.P.: data collection, data interpre- tation, draft manuscript preparation, substantial manuscript preparation. G.N.: data collection, data interpretation. E.B.: data collection, data interpretation, final manuscript revision. V.G.D.: project concept, project design, final manuscript revision. D.P.M.: project concept, project design, final manuscript revision. S.I.L.: project lead, funding, resources, project concept, project design, substantial manuscript preparation, final manuscript revision. All authors contributed to the article and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK-03762).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author/s.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could implicate potential conflict of interest.

REFERENCES

Ghorbani, A.; Esmaeilizadeh, M. Pharmacological properties of *Salvia officinalis* and its components. J. Tradit. Complement. Med.2017, 7, 433–440. [CrossRef]

Hamidpour, M.; Hamidpour, R.; Hamidpour, S.; Shahlari, M. Chemistry, pharmacology, and medicinal property of sage (salvia) to prevent and cure illnesses such as obesity, diabetes, depression, dementia, lupus, autism, heart disease, and cancer. J. Tradit. Complement. Med. 2014, 4, 82–88. [CrossRef]

Lu, Y.; Foo, L.Y. Polyphenolics of Salvia—A review. Phytochemistry 2002, 59, 117–140. [CrossRef]

Sharma, Y.; Fagan, J.; Schaefer, J. Ethnobotany, phytochemistry, cultivation and medicinal properties of Garden sage (*Salvia officinalis* L.). J. Pharmacogn. Phytochem. 2019, 8, 3139–3148.

Ben Farhat, M.; Chaouch-Hamada, R.; Sotomayor, J.A.; Landoulsi, A.; Jordan, M.J. Antioxidant potential of *Salvia officinalis* L. residues as affected by the harvesting time. Ind. Crops Prod. 2014, 54, 78–85. [CrossRef]

Glisic, S.B.; Ristic, M.; Skala, D.U. The combined extraction of sage (*Salvia officinalis* L.): Ultrasound followed by supercritical CO2 extraction. Ultrason. Sonochem. 2011, 18, 318–326. [CrossRef]

Martins, N.; Barros, L.; Santos-Buelga, C.; Henriques, M.; Silva, S.; Ferreira, I.C. Evaluation of bioactive properties and phenolic compounds in different extracts prepared from *Salvia officinalis* L. Food Chem. 2015, 170, 378–385. [CrossRef]

Nutrizio, M.; Kljusuric´, J.; Sabolovic´, M.; Kovacˇevic´, D.; Šupljika, F.; Putnik, P.; Cˇ akic´, M.; Dubrovic´, I.; Vrsaljko, D.; Maltar-

Strmecˇki, N.; et al. Valorization of sage extracts (*Salvia officinalis* L.) obtained by high voltage electrical discharges: Process control and antioxidant properties. Innov. Food Sci. Emerg. Technol. 2020, 60, 102284. [CrossRef]

Pavlic´, B.; Vidovic´, S.; Vladic´, J.; Radosavljevic´, R.; Cindric´, M.; Zekovic´, Z. Subcritical water extraction of sage (*Salvia officinalis* L.) by-products—Process optimization by response surface methodology. J. Supercrit. Fluids 2016, 116, 36–45. [CrossRef]

Putnik, P.; Lorenzo, J.M.; Barba, F.J.; Roohinejad, S.; Režek Jambrak, A.; Granato, D.; Montesano, D.; Bursac´ Kovacˇevic´, D. Novel food processing and extraction technologies of high-added value compounds from plant materials. Foods 2018, 7, 106. [CrossRef] [PubMed]

Shirazi, S.; Nia Ahmad, P.; Asl Mohammad Reza, S.; Naghipour, F.; Tavakolipour, H. Antioxidant activity of aqueous and alcohol extracts of Salvia leriifolia L. and Linum usitalissmum L. subjected to a pulsed electric field. Foods Raw Mater. 2020, 8, 186–195. [CrossRef]

Sosa Ferrera, Z.; Padrón Sanz, C.; Mahugo Santana, C.; Santana Rodríguez, J.J. The use of micellar systems in the extraction and pre-concentration of organic pollutants in environmental samples. TrAC Trends Anal. Chem. 2004, 23, 469–479. [CrossRef]

Asami, D.K.; Hong, Y.J.; Barrett, D.M.; Mitchell, A.E. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. J. Agric. Food Chem. 2003, 51, 1237–1241. [CrossRef] [PubMed]

Mueller-Harvey, I. Analysis of hydrolysable tannins. Anim. Feed Sci. Technol. 2001, 91, 3–20. [CrossRef]

Chemat, F.; Rombaut, N.; Meullemiestre, A.; Turk, M.; Perino, S.; Fabiano-Tixier, A.-S.; Abert-Vian, M. Review of green food processing techniques. Preservation, transformation, and extraction. Innov. Food Sci. Emerg. Technol. 2017, 41, 357–377. [CrossRef]

Álvarez, I.; Pagán, R.; Condon, S.; Raso, J. The influence of process parameters for the inactivation of Listeria monocytogenes by pulsed electric fields. Int. J. Food Microbiol. 2003, 87, 87–95. [CrossRef]

Grahl, T.; Märkl, H. Killing of microorganisms by pulsed electric fields. Appl. Microbiol. Biotechnol. 1996, 45, 148–157. [CrossRef]

Bobinaite˙, R.; Pataro, G.; Lamanauskas, N.; Šatkauskas, S.; Viškelis, P.; Ferrari, G. Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products. J. Food Sci. Technol. 2015, 52, 5898–5905. [CrossRef]

Carullo, D.; Pataro, G.; Ferrari, G. Effect of PEF pre-treatment and extraction temperature on the recovery of carotenoids from tomato wastes. Chem. Eng. Trans. 2019, 75, 139–144. [CrossRef]

Drosou, F.; Yang, E.; Marinea, M.; Dourtoglou, T.; Chatzilazarou, A.; Dourtoglou, V. An assessment of potential applications with pulsed electric field in wines. BIO Web Conf. 2017, 9, 02010. [CrossRef]

Martín, B.; Tylewicz, U.; Verardo, V.; Pasini, F.; Caravaca, A.M.G.; Caboni, M.; Dalla Rosa, M. Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers' spent grains. Innov. Food Sci. Emerg. Technol. 2020, 64, 102402. [CrossRef]

Pataro, G.; Carullo, D.; Siddique, A.; Falcone, M.; Donsì, F.; Ferrari, G. Improved extractability of carotenoids from tomato peels as side benefits of PEF treatment of tomato fruit for more energy-efficient steam-assisted peeling. J. Food Eng. 2018, 233, 65–73. [CrossRef]

Tsapou, E.A.; Ntourtoglou, G.; Drosou, F.; Tataridis, P.; Dourtoglou, T.; Lalas, S.; Dourtoglou, V. In situ creation of the natural phenolic aromas of beer: A pulsed electric field applied to wort-enriched flax seeds. Front. Bioeng. Biotechnol. 2020, 8, 1219. [CrossRef] [PubMed]

Tzima, K.; Brunton, N.P.; Lyng, J.G.; Frontuto, D.; Rai, D.K. The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products. Innov. Food Sci. Emerg. Technol. 2021, 69, 102644. [CrossRef]

Bozinou, E.; Karageorgou, I.; Batra, G.; Dourtoglou, V.; Lalas, S. Pulsed electric field extraction and antioxidant activity determination of Moringa oleifera dry leaves: A comparative study with other extraction techniques. Beverages 2019, 5, 8. [CrossRef]

Brodelius, P.E.; Funk, C.; Shillito, R.D. Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored secondary products. Plant. Cell Rep. 1988, 7, 186–188. [CrossRef] [PubMed]

Pappas, V.M.; Lakka, A.; Palaiogiannis, D.; Bozinou, E.; Ntourtoglou, G.; Batra, G.; Athanasiadis, V.; Makris, D.P.; Dourtoglou, V.G.; Lalas, S.I. Use of Pulsed Electric Field as a low-temperature and high-performance “green” extraction technique for the recovery of high added value compounds from olive leaves. Beverages 2021, 7, 45. [CrossRef]

Lakka, A.; Grigorakis, S.; Karageorgou, I.; Batra, G.; Kaltsa, O.; Bozinou, E.; Lalas, S.; Makris, D.P. Saffron processing wastes as a bioresource of high-value added compounds: Development of a green extraction process for polyphenol recovery using a natural deep eutectic solvent. Antioxidants 2019, 8, 586. [CrossRef]

Kaltsa, O.; Grigorakis, S.; Lakka, A.; Bozinou, E.; Lalas, S.; Makris, D. Green valorization of olive leaves to produce polyphenol- enriched extracts using an environmentally benign deep eutectic solvent. AgriEngineering 2020, 2, 14. [CrossRef]

Hjelmeland, A.K.; King, E.S.; Ebeler, S.E.; Heymann, H. Characterizing the chemical and sensory profiles of United States Cabernet Sauvignon wines and blends. Am. J. Enol. Vitic. 2013, 64, 169–179. [CrossRef]

Gabric´, D.; Barba, F.; Roohinejad, S.; Gharibzahedi, S.M.T.; Radojc˘in, M.; Putnik, P.; Bursac´ Kovac˘evic´, D. Pulsed electric fields as an alternative to thermal processing for preservation of nutritive and physicochemical proper-ties of beverages: A review. J. Food Process. Eng. 2018, 41, e12638. [CrossRef]

Batra, G.; Gortzi, O.; Lalas, S.I.; Galidi, A.; Alibade, A.; Nanos, G.D. Enhanced antioxidant activity of Capsicum annuum L. and

Moringa oleifera L. extracts after encapsulation in microemulsions. ChemEngineering 2017, 1, 15. [CrossRef]

Kozłowska, M.; Gruczyn´ska, E. Comparison of the oxidative stability of soybean and sunflower oils enriched with herbal plant extracts. Chem. Pap. 2018, 72, 2607–2615. [CrossRef]

Sgorbini, B.; Bicchi, C.; Cagliero, C.; Cordero, C.; Liberto, E.; Rubiolo, P. Herbs and spices: Characterization and quantitation of biologically-active markers for routine quality control by multiple headspace solid-phase microextraction combined with separative or non-separative analysis. J. Chromatogr. A 2015, 1376, 9–17. [CrossRef]

Pistelli, L.; Noccioli, C.; D’Angiolillo, F.; Pistelli, L. Composition of volatile in micropropagated and field grown aromatic plants from Tuscany Islands. Acta Biochim. Pol. 2013, 60, 43–50. [CrossRef]

D’Auria, M.; Racioppi, R. The effect of drying of the composition of volatile organic compounds in Rosmarinus officinalis, Laurus nobilis, *Salvia officinalis* and Thymus serpyllum. A HS-SPME-GC-MS study. J. Essent. Oil Bear. Plants 2015, 18, 1209–1223. [CrossRef]

Venskutonis, P.R. Effect of drying on the volatile constituents of thyme (Thymus vulgaris L.) and sage (*Salvia officinalis* L.). Food Chem. 1997, 59, 219–227. [CrossRef]

Jokic´, S.; Molnar, M.; Jakovljevic´, M.; Aladic´, K.; Jerkovic´, I. Optimization of supercritical CO2 extraction of *Salvia officinalis*

L. leaves targeted on Oxygenated monoterpenes, α-humulene, viridiflorol and manool. J. Supercrit. Fluids 2018, 133, 253–262. [CrossRef]

Tundis, R.; Leporini, M.; Bonesi, M.; Rovito, S.; Passalacqua, N.G. *Salvia officinalis* L. from Italy: A Comparative Chemical and Biological Study of Its Essential Oil in the Mediterranean Context. Molecules 2020, 25, 5826. [CrossRef] [PubMed]

El Ouadi, Y.; Manssouri, M.; Bouyanzer, A.; Majidi, L.; Lahhit, N.; Bendaif, H.; Hammouti, B. Essential oil composition and antifungal activity of *Salvia officinalis* originating from North-East Morocco, against postharvest phytopathogenic fungi in apples. Pharma Chem. 2015, 7, 95–102.

Sotelo, K.A.G.; Hamid, N.; Oey, I.; Gutierrez-Maddox, N.; Ma, Q.; Leong, S.Y. Effect of pulsed electric fields on the flavour profile of red-fleshed sweet cherries (Prunus avium var. Stella). Molecules 2015, 20, 5223–5238. [CrossRef]

Atwi, M.; Weiss, E.K.; Loupassaki, S.; Makris, D.P.; Ioannou, E.; Roussis, V.; Kefalas, P. Major antioxidant polyphenolic phytochemicals of three salvia species endemic to the island of Crete. J. Herbs Spices Med. Plants 2016, 22, 27–34. [CrossRef]

Zimmermann, B.F.; Walch, S.G.; Tinzoh, L.N.; Stühlinger, W.; Lachenmeier, D.W. Rapid UHPLC determination of polyphenols in aqueous infusions of *Salvia officinalis* L. (sage tea). J. Chromatogr. B 2011, 879, 2459–2464. [CrossRef]

Mylonaki, S.; Kiassos, E.; Makris, D.P.; Kefalas, P. Optimisation of the extraction of olive (Olea europaea) leaf phenolics using water/ethanol-based solvent systems and response surface methodology. Anal. Bioanal. Chem. 2008, 392, 977–985. [CrossRef] [PubMed]

Bljajic, K.; Brajkovic, A.; Cacic, A.; Vujic, L.; Jablan, J.; Saraiva de Carvalho, I.; Zovko Koncic, M. Chemical Composition, Antioxidant, and a-Glucosidase-Inhibiting Activity of Aqueous and Hydroethanolic Extracts of Traditional Antidiabetics from Croatian Ethnomedicine. Horticulturae 2021, 7, 15. [CrossRef]

Niu, D.; Zeng, X.A.; Ren, E.F.; Xu, F.Y.; Li, J.; Wang, M.S.; Wang, R. Review of the application of pulsed electric fields (PEF) technology for food processing in China. Food Res. Int. 2020, 137, 109715. [CrossRef]

Δημοσίευση VII

Hyphenated Extraction of Valuable Compounds from *Aesculus carnea*: Ultrasound Extraction with Pulsed Electric Field Pretreatment

George Ntourtoglou[1], Fotini Drosou[1], Vassilis G. Dourtoglou[1], Vassilis Athanasiadis[2], Theodoros Chatzimitakos [2], Eleni Bozinou [2] and Stavros I. Lalas [2,*]

1. Department of Wine, Vine, and Beverage Sciences, School of Food Science, University of West Attica, 12243 Athens, Greece
2. Department of Food Science and Nutrition, University of Thessaly, 43100 Karditsa, Greece

*

Keywords: *Aesculus carnea*; HPLC-DAD; hydroethanolic solution; phenolic compounds; pulsed electric field; ultrasound-assisted extraction

ABSTRACT

Wood-related procedures, such as lumberjacking and pruning, inevitably result in big piles of leaves, which are considered a major by-product. Extracting valuable compounds from natural by-products is an ongoing trend. In this work, the use of Pulsed Electric Field (PEF) was evaluated as a pretreatment step, prior to the ultrasound-assisted extraction of phenolic compounds from *Aesculus carnea* leaves. In addition, various solvent systems were examined, as well as the time of pretreatment with PEF. According to the results, up to 33% more phenolic compounds can be extracted, under optimum conditions (30% ethanol in water as solvent and PEF pretreatment for 30 min, compared to the same solvent, without PEF). Moreover, PEF treatment time was not (i.e., 30 and 60 min) and no differences were recorded, suggesting that a lower treatment time can yield the same extraction of phenolic compounds. As such, the use of PEF is highly recommended in combination with ultrasound extraction, to maximize the yield of phenolic compounds extracted from the leaves of *Aesculus carnea*.

INTRODUCTION

Aesculus x carnea (Family: Sapindaceae, species: *Aesculus carnea*, Hayne) commonly known as European horse chestnut is a large tree native to the Balkans forests of Europe. It can grow up to 40 m and it has long leaves around 15–30 cm. It is known that its leaves are a rich source of phenolics [1,2]. It was not until recently that studies have showcased that compounds found in Aesculus spp. exhibit even more benefits than initially believed [2]. Polyphenolic compounds derive naturally, as a result of the plant metabolism and more specifically from the acetate pathway or the shikimate pathway [1]. Polyphenolics can have a wide variety of chemical structures (and therefore, size, weight etc.), and can be composed of different monomer units [3]. As regards the monomer units of the polyphenolic compounds, there is a wide variety of monomers, resulting in an even bigger variety of polymer structures [4]. Despite the fact that polyphenolic compounds can be found free in plant tissues, usually, polyphenolic conjugates with other compounds can be found. For instance, carbohydrate sugars are one such class of compounds that usually forms conjugates with polyphenolics. Conjugation is achieved mainly via the hydroxyl group of the polyphenolic compound that usually binds to the sugar residues naturally present in the plant. Such compounds can vary in chain length and composition. However, glucose is most often conjugated with polyphenolics due to its prevalence in plant physiology. While plant sugar residues can bind to any of the compound's aromatic carbons, this is not the most common form of attachment [4,5]. One important sub-class of polyphenolic compounds is flavonols. They are a well-known group of compounds with

pronounced antioxidant and ultraviolet light absorption properties [6]. Moreover, recently, their anticancer activity was also highlighted [7].

Up to now, many techniques have been employed to extract bioactive compounds from plants. However, there is still an increased interest in developing new, advanced procedures, to overcome the disadvantages of the existing techniques. One such new technique is the pulsed electric field (PEF). PEF is a green technique, relatively new that can be used to enhance the extraction processes [8]. It has been proven that by using PEF in the extraction process the total amount of specific compounds, especially polyphenols, can be increased [9,10]. The PEF typically is used before the main extraction step, as pre-treatment. Typically, short-duration pulses between (100 ns–1 ms) are used with a voltage between 1 kV and 3 kV [11]. So far, PEF has been used for the enhancement of the extraction of polyphenolics from citrus fruits [12], potato peels [13], Sideritis plants [14], rapeseed stems [13], etc. However, there were reported cases where the use of PEF did not increase the total polyphenol content (TPC) of other plants, such as Thymus serpyllum [15]. Therefore, its benefits should not be taken for granted, and further exploited on specific cases.

This work aims to examine the combinatorial effect of PEF (used as a pre-treatment step) along with ultrasound on the extraction of the phenolic compounds from *A. carnea*. The extraction was carried out using a simple, ultrasound-assisted procedure. Various solvent systems and PEF treatment times were examined prior ultrasound treatment, so as to examine whether the use of PEF is beneficial for polyphenol extraction.

MATERIALS AND METHODS

Chemicals

Absolut ethanol, acetonitrile and formic acid were obtained from Carlo Erba (Val de Reuil, France). Glycerol anhydrous, gallic acid monohydrate and anhydrous sodium carbonate (>99%) were purchased from Penta (Prague, Czech Republic). Folin–Ciocalteu reagent was obtained from Panreac (Barcelona, Spain). Chemical standards for the HPLC- based determination of polyphenols (i.e., neochlorogenic acid, kaempferol 3-O-β-rutinoside, kaempferol 3-glucoside, quercetin 3-O-galactoside, quercetin and kaempferol) and polypropy- lene glycol were purchased from Sigma-Aldrich (Steinheim, Germany). A deionizing column was utilized to create the deionized water that was used in the experiments. All the chromatography solvents utilized were HPLC grade.

Plant Material Preparation and Extraction

Fresh leaves from a 20 year old *A. carnea* tree were collected in Afidnes area (Attica, Greece, at according to Google Earth version 7.3.2.5776 Latitude: 38.181001 and Longitude: 23.849508) (the moisture of the leaves was calculated to be 75 3%). Then, the leaves were washed thoroughly with deionized water and dried with paper towels. Next, 10 g of the leaves were cut into smaller pieces (<1 cm). Half of the leaves (5 g) were placed in the PEF treatment chamber, while the remaining quantity was left in the beaker (control sample). After 60 min, the PEF-treated leaves were removed from the chamber and placed in a glass beaker. Similarly, the non-PEF-treated leaves were also placed in a similar glass beaker. In both cases, 50 mL of an appropriate solvent was added [the solvents examined were: (I) deionized water, (II) 30% ethanol in water, (III) 30% glycerol in water and (IV) 30% polypropylene glycol in water]. The solid-to-liquid ratio employed herein resulted from our preliminary experiments, exhibiting a 18% increase compared to 1:5 ratio and 27% compared to 1:1 ratio. Next, the beakers were placed in an ultrasonic bath for 15 min. Then, the samples were centrifuged at 4000 rpm for 5 min and filtered using 0.45 μm filter papers. The same procedure was followed to determine the effect of PEF treatment time, by altering the treatment time (30 min or 60 min). In this case, the solvent used for ultrasound- assisted extraction was 30% ethanol in water. Finally, the extracts were injected into the HPLC system and further analyzed.

Instrumentation

The PEF instrument used is given in detail in our previous studies [14]. Briefly, the system consisted of a high-voltage current generator (Leybold, LD Didactic GmbH, Hürth, Germany), a digital oscilloscope, a function/arbitrary waveform generator, and two stainless steel plates (10 cm long, 10 cm high) with 1 cm Teflon between them, for insulation. The pulse duration used to process the sample was 1 μs, the pulse frequency was 1 Hz, the electric field strength was 1 kV cm−1, the waveform was a typical square wave, and the maximum delay was less than 20 ns. PEF parameters were selected based on our previous studies [10,13]. Ultrasound treatment of the samples was carried out in a Transonic 570/H (ELMA) unit, with a frequency of 35 kHz, and a high-frequency peak of 320 W.

The HPLC system used in this study was a Shimadzu CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany), connected to a diode array detector (Shimadzu SPD-M20A). A Phenomenex Luna C18 column (5 μm, 4.6 mm 250 mm) (Phenomenex Inc., Torrance, CA, USA) was used as a stationary phase. The column was thermostated at 40 ◦C, during separation. The mobile phase consisted of (A) water containing 0.5% v/v formic acid and (B) a mixture of acetonitrile: water (60:40) containing 0.5% v/v formic acid. The gradient program was as follows: 5% B to 40% B in 40 min, then to 50% B in 10 min, and finally to 70% B in 10 min and kept constant for 10 more minutes. The flow rate was set at 1 mL min−1. The total program run time was 70 min. The injection volume was 20 μL and injections were made using a rheodyne injector. Spectra were recorded between 220 and 360 nm. Identification of the compounds was carried out by comparing the retention time with that of standard compounds, as well as the absorbance spectra. For the quantification of the compounds, calibration curves were prepared with standard compounds and using the equations, the concentration of the compounds in the samples was determined.

Comparative Analysis of Total Polyphenol Content (TPC) of the Extracts

The TPC of the extracts was determined using the Folin–Ciocalteu assay, as previously described [16]. In brief, 0.1 mL of the diluted sample extract was mixed with an equal amount of the Folin–Ciocalteu reagent. After 2 min of incubation, 0.8 mL sodium carbonate aqueous solution (5% w/v) was added, and the solution was incubated for 20 min at 40 ◦C. Finally, the absorbance of the solution was measured at 740 nm. For the expression of the results in gallic acid equivalents (GAE), a calibration curve was prepared with gallic acid.

Statistical Analysis

All experiments were carried out three times and each sample was measured in triplicate, and therefore the results are expressed as mean values of all measurements, and variability of the results was expressed using the standard deviation (mean value standard deviation). Statistically significant differences between control samples and treated samples were evaluated with a t-test (after testing for normality of data with the Shapiro–Wilk test) for $p < 0.05$, using SPSS (version 26) (SPSS Inc., Chicago, IL, USA) software.

RESULTS AND DISCUSSION

Since *A. carnea* can be commonly found in many cities as an ornamental plant, the valorization of its leaves in order to isolate bioactive compounds would be of high interest [1,2]. The most common method to prepare an extract is ultrasound treatment. However, PEF may have the potential to enhance the extraction yield, in an environmentally friendly way. To this end, the combinatory efficiency of PEF prior to ultrasound treatment was examined and optimized.

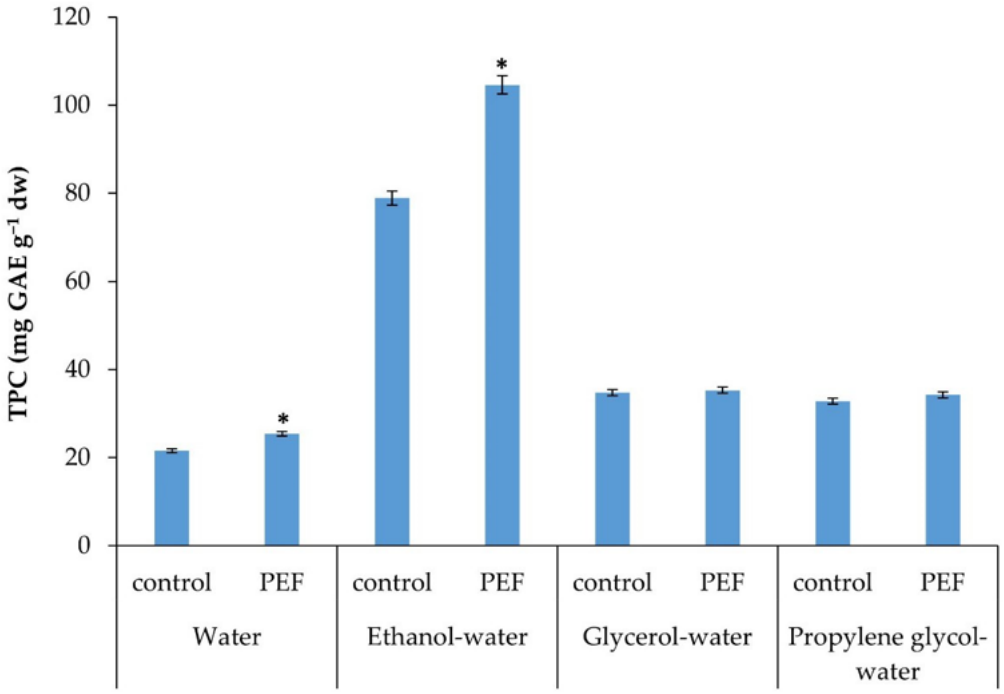


Figure 1. Total polyphenols, TPC (mg GAE g^{-1} dw), of the extracts after reaction with the Folin-Ciocalteu reagent; Statistically significant differences are denoted with an asterisk (*) for $p < 0.05$; Error bars denote the standard deviation of nine replicate analyses.

In order to assess the efficiency of the various solvents used for preparing the extracts of the plant, as well as whether the use of PEF as a pre-treatment step can further assist the extraction of bioactive compounds, the Folin–Ciocalteu assay was used. In this assay, the polyphenolic compounds react with the reagent and yield products that can be measured with photometers. In this context, the absorbance of the plant extracts prepared with and without PEF, after reaction with the Folin–Ciocalteu reagent was used as a criterion to evaluate and compare the performance of the various conditions. Results are presented in Figure 1 and expressed as mg GAE per g of dry weight (mg GAE g−1 dw). As indicated by the results, the control samples (prepared without PEF pre-treatment) the optimum extraction solvent was the 30% ethanol in water mixture. The rest of the solvents, the glycerol-water and the propylene glycol-water mixtures yielded ~60% better results compared to plain water, but still less than the ethanol-water mixture. As regards the pre-treatment of the samples with PEF (60 min), it can be seen that, in the cases of water and ethanol-water mixture, the PEF pre-treated samples yielded higher results, that were found to be statistically significant for $p < 0.05$. The smaller increase (~4.6%) was recorded for the propylene glycol–water mixture, whereas when plain water was used a 18% increase was recorded. However, the most notable increase was recorded in the case of the ethanol–water mixture, where the yield of polyphenolic compounds was increased by 33%. As regards the solvent used, they were selected due to their potential to extract phenolic compounds. Ethanol, not only is a commonly employed solvent for the extraction of polyphenolic compounds, but there are also many reports that highlight the superiority of hydroethanolic mixtures over water for the extraction of the compounds [17–19]. Glycerol is a naturally occurring

compound that is also produced during biodiesel production, widely employed in food industry [20]. Due to its hydrophilic properties, it can be dissolved in water, and is considered as a more “green” alternative to ethanol. Glycerol has also been employed for the extraction of polyphenols from various plants, and in some cases, glycerol–water mixtures were found more efficient compared to ethanol-water mixtures [20–22]. Another polyol that has been studied for its potential for polyphenol extraction is propylene glycol. It is a safe compound for human consumption and has been used for the isolation of polyphenols from Camellia seeds [23], Medicago lupulina L. [24] and others [25]. Among the solvents used, the presence of ethanol was found to be beneficial. This is in accordance with previous reports [14,17–19,26,27]. A common explanation would be that the presence of ethanol in the extraction medium decreases the polarity of the solvent, rendering it more suitable to extract less polar compounds. However, this is also the case with the examined polyols, rendering this explanation insufficient to explain the observed results. Moreover, it would also be expected that polyols would achieve a higher extraction efficiency, since they are capable of forming more hydrogen bonds with the polyphenols, compared to ethanol [22]. The polyol–water mixtures have increased viscosity, compared to plain water, since polyols, form an extensive network of hydrogen bonds, ascribing them high viscosity [20]. Thus, although the polyol–water mixtures are less polar than water and able to form more hydrogen bonds with the polyphenols, they have lower permeability to the plant tissue, due to their high viscosity. Ethanol addition in the water increases the permeability of cells, making easier the transfer of the compounds towards the extraction medium. Finally, it increases the heat conductivity of the solution, resulting in better heat transfer towards the cells and as a result, enhances the overall extraction procedure [28]. Regarding the use of PEF, there are previous studies that report increased extraction yield of polyphenols after treatment of the samples with PEF [14,29–31]. However, this is not always the case, since there are reports that the PEF treatment exhibits limited benefits for the overall extraction. For instance, in the study of Pollini et al. [32] the PEF pre-treatment of fresh apple pomace did not increase the extraction yield of polyphenol compounds. Similarly, in the study of Carpentieri et al. [33] the use of PEF prior to extraction of oregano and thyme with a hydroethanolic solution resulted in a nearly 7% increase in the TPC. Therefore, our results highlight the benefits of PEF usage, as a pre-treatment step to obtain extracts from *A. carnea* leaves that contain more polyphenols.

Based on the abovementioned results, we further examined whether a shorter time of

PEF treatment (i.e., 30 min) would result in a similar content in polyphenols, compared to the treatments for 60 min, using the optimum solvent system. However, at this point, evaluation was based on individual polyphenolic components (presented in Table 1), and not on the TPC, in an effort to examine whether there are differences among the individual components, or if they all exhibit similar behavior. Representative chromatograms of the extract are presented in Figure 2.

In the case of pretreatment with PEF for 30 min, the content of polyphenols in the

Table 1. Content of specific polyphenols in the extracts (expressed as mean values ± standard deviation of nine replicate analyses) extracted with US only (without PEF) and after pretreatment with PEF for 30 or 60 min; Statistically significant differences are denoted with capital and small letters for $p < 0.05$.

Compound	Polyphenol Content (mg g^{-1})		
	Without PEF	PEF 30 min	PEF 60 min
Neochlorogenic acid	0.42 ± 0.03 ^{A}	0.52 ± 0.05 ^{a}	0.51 ± 0.06 ^{a}
Kaempferol 3-*O*-β-rutinoside	18 ± 1 ^{A}	22 ± 2 ^{a}	24 ± 3 ^{a}
Kaempferol 3-glucoside	28 ± 2 ^{A}	35 ± 3 ^{a}	40 ± 4 ^{a}
Quercetin 3-*O*-galactoside	0.23 ± 0.02 ^{A}	0.29 ± 0.03 ^{a}	0.36 ± 0.04 ^{a}
Quercetin	1.7 ± 0.2 ^{A}	2.4 ± 0.2 ^{a}	2.8 ± 0.3 ^{a}
Kaempferol	1.8 ± 0.2 ^{A}	2.6 ± 0.3 ^{a}	3.1 ± 0.3 ^{a}
Total identified	*50.2*	*62.8*	*70.7*

extract was found to be 62.8 mg g−1, whereas in the case of pretreatment with PEF for 60 min, the content of polyphenols in the extract was found to be 70.7 mg g−1. No statistically significant differences ($p > 0.05$) were recorded for each examined compound, between the different samples, signifying that pretreatment with PEF for a shorter period is also able to achieve the same outcome, thus, further reducing the time and cost of the extraction process, without having a toll on the total extraction yield. Moreover, since no notable differences were recorded, it can be assumed that the PEF pretreatment assists the extraction process in a non-specific way, without being affected by the physicochemical properties of specific compounds (e.g., log Kow).

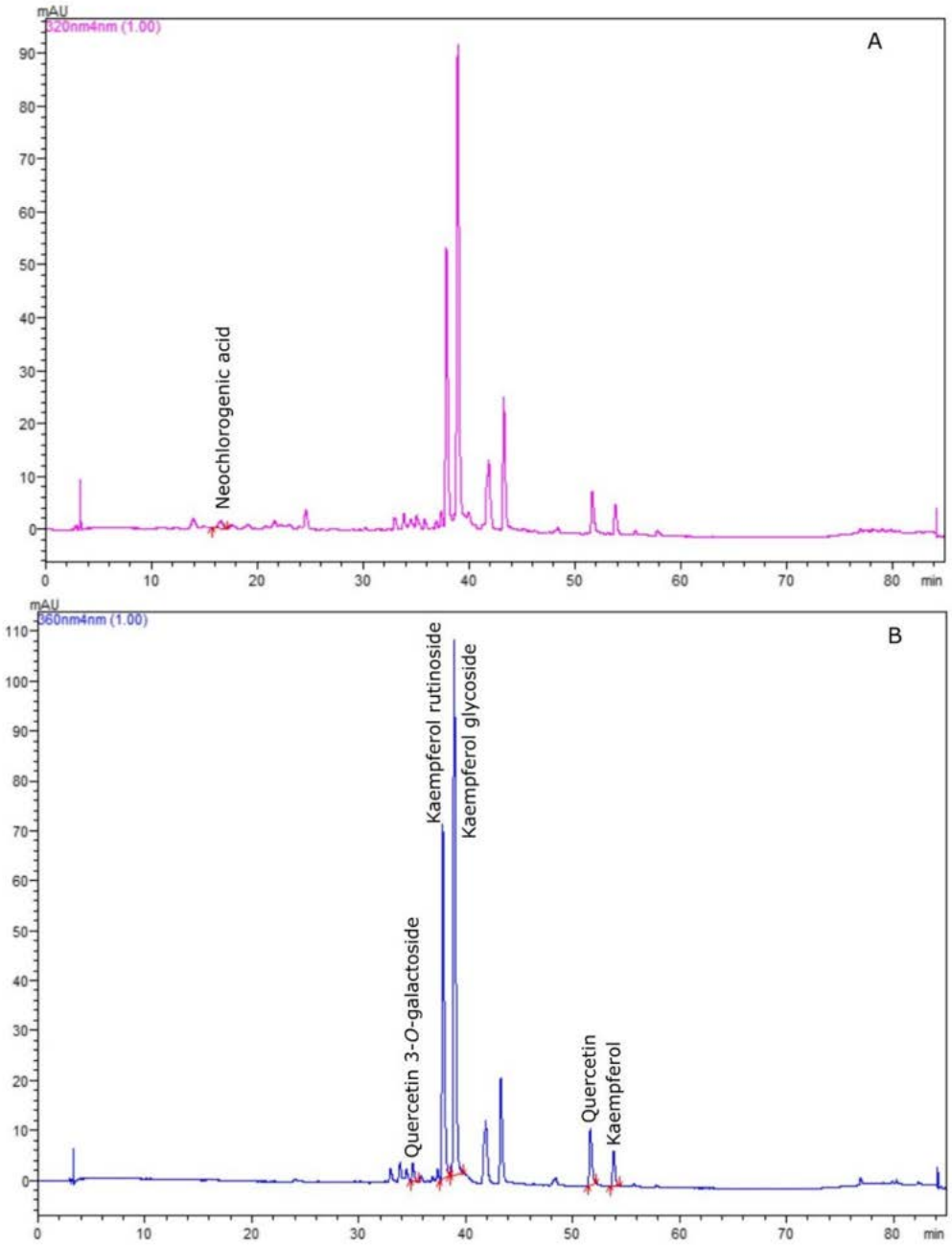


Figure 2. Representative chromatograms of the *A. carnea* extract at (**A**) 320 nm and (**B**) 360 nm.

CONCLUSIONS

In this study, the benefit of using PEF as a pretreatment, prior to US extraction of polyphenols from *A. carnea* was showcased. A significant enhancement of up to ~33% was recorded when PEF was employed. This percentage was achieved using an ethanol–water mixture as a solvent, which also enhanced the extraction of polyphenols,

compared to plain water and polyol–water mixtures (i.e., glycerol and propylene glycol). The individual compounds examined exhibited a similar extractability for different PEF pretreatment times, suggesting a non-specific extraction mechanism, less dependent on the physicochemical properties of the specific polyphenols. The above data suggest that the proposed method can be used to enhance the content of polyphenols in the extracts, in a cost-efficient way.

Author Contributions: Conceptualization, S.I.L. and V.G.D.; methodology, T.C., G.N. and V.A.; software, T.C.; validation, V.A., T.C., G.N. and E.B.; formal analysis, G.N., F.D., V.A., T.C. and E.B.; investigation, G.N., F.D., V.A., T.C. and E.B.; resources, V.G.D. and S.I.L.; data curation, G.N., F.D., V.A., T.C. and E.B.; writing—original draft preparation, G.N. and F.D.; writing—review and editing, all authors; visualization, T.C.; supervision, S.I.L. and V.G.D.; project administration, S.I.L.; funding acquisition, S.I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: Authors would like to thank Maria Alexandri (Agronomist, Vioryl Chemical & Agricultural Industry, Research S.A.) for the identification of the tree specimen.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Oszmian´ski, J.; Kalisz, S.; Aneta, W. The Content of Phenolic Compounds in Leaf Tissues of White (Aesculus hippocastanum L.) and Red Horse Chestnut (Aesculus carea H.) Colonized by the Horse Chestnut Leaf Miner (Cameraria ohridella Deschka & Dimic´). Molecules 2014, 19, 14625–14636. [CrossRef]

Idris, S.; Mishra, A.; Khushtar, M. Phytochemical, ethanomedicinal and pharmacological applications of escin from Aesculus hippocastanum L. Towards future medicine. J. Basic Clin. Physiol. Pharmacol. 2020, 31, 20190115. [CrossRef]

Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. Am. J. Clin. Nutr.2004, 79, 727–747. [CrossRef]

Abbas, M.; Saeed, F.; Anjum, F.M.; Afzaal, M.; Tufail, T.; Bashir, M.S.; Ishtiaq, A.; Hussain, S.; Suleria, H.A.R. Natural polyphenols: An overview. Int. J. Food Prop. 2017, 20, 1689–1699. [CrossRef]

Ebrahimi, P.; Lante, A. Polyphenols: A Comprehensive Review of their Nutritional Properties. Open Biotechnol. J. 2021, 15,164–172. [CrossRef]

Laoué, J.; Fernandez, C.; Ormeño, E. Plant Flavonoids in Mediterranean Species: A Focus on Flavonols as Protective Metabolites under Climate Stress. Plants 2022, 11, 172. [CrossRef]

Kubina, R.; Iriti, M.; Kabała-Dzik, A. Anticancer Potential of Selected Flavonols: Fisetin, Kaempferol, and Quercetin on Head and Neck Cancers. Nutrients 2021, 13, 845. [CrossRef]

Ranjha, M.M.A.N.; Kanwal, R.; Shafique, B.; Arshad, R.N.; Irfan, S.; Kieliszek, M.; Kowalczewski, P.; Irfan, M.; Khalid, M.Z.; Roobab, U.; et al. A Critical Review on Pulsed Electric Field: A Novel Technology for the Extraction of Phytoconstituents. Molecules 2021, 26, 4893. [CrossRef]

Arshad, R.N.; Abdul-Malek, Z.; Roobab, U.; Qureshi, M.I.; Khan, N.; Ahmad, M.H.; Liu, Z.; Aadil, R.M. Effective valorization of food wastes and by-products through pulsed electric field: A systematic review. J. Food Process Eng. 2021, 44, e13629. [CrossRef]

Soliva-Fortuny, R.; Balasa, A.; Knorr, D.; Martín-Belloso, O. Effects of pulsed electric fields on bioactive compounds in foods: A review. Trends Food Sci. Technol. 2009, 20, 544–556. [CrossRef]

Lakka, A.; Bozinou, E.; Makris, D.; Lalas, S. Evaluation of Pulsed Electric Field Polyphenol Extraction from Vitis vinifera, Sideritis scardica and Crocus sativus. ChemEngineering 2021, 5, 25. [CrossRef]

EL Kantar, S.; Boussetta, N.; Lebovka, N.; Foucart, F.; Rajha, H.N.; Maroun, R.G.; Louka, N.; Vorobiev, E. Pulsed electric field treatment of citrus fruits: Improvement of juice and polyphenols extraction. Innov. Food Sci. Emerg. Technol. 2018, 46, 153–161. [CrossRef]

Frontuto, D.; Carullo, D.; Harrison, S.M.; Brunton, N.P.; Ferrari, G.; Lyng, J.G.; Pataro, G. Optimization of Pulsed Electric Fields-Assisted Extraction of Polyphenols from Potato Peels Using Response Surface Methodology. Food Bioprocess Technol. 2019, 12, 1708–1720. [CrossRef]

Athanasiadis, V.; Pappas, V.M.; Palaiogiannis, D.; Chatzimitakos, T.; Bozinou, E.; Makris, D.P.; Lalas, S.I. Pulsed Electric Field- Based Extraction of Total Polyphenols from Sideritis raiseri Using Hydroethanolic Mixtures. Oxygen 2022, 2, 91–98. [CrossRef]

Tzima, K.; Brunton, N.P.; Lyng, J.G.; Frontuto, D.; Rai, D.K. The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products. Innov. Food Sci. Emerg. Technol. 2021, 69, 91–98. [CrossRef]

Ainsworth, E.A.; Gillespie, K.M. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nat. Protoc. 2007, 2, 875–877. [CrossRef]

Gironi, F.; Piemonte, V. Temperature and solvent effects on polyphenol extraction process from chestnut tree wood. Chem. Eng. Res. Des. 2011, 89, 857–862. [CrossRef]

Imeneo, V.; Romeo, R.; De Bruno, A.; Piscopo, A. Green-sustainable extraction techniques for the recovery of antioxidant compounds from “citrus Limon” by-products. J. Environ. Sci. Health Part B Pestic. Food Contam. Agric. Wastes 2022, 57, 220–232. [CrossRef]

Peiró, S.; Gordon, M.H.; Blanco, M.; Pérez-Llamas, F.; Segovia, F.; Almajano, M.P. Modelling Extraction of White Tea Polyphenols: The Influence of Temperature and Ethanol Concentration. Antioxidants 2014, 3, 684–699. [CrossRef]

Makris, D.P.; Lalas, S. Glycerol and Glycerol-Based Deep Eutectic Mixtures as Emerging Green Solvents for Polyphenol Extraction: The Evidence So Far. Molecules 2020, 25, 5842. [CrossRef]

Kowalska, G.; Baj, T.; Kowalski, R.; Szyman´ska, J. Optimization of Glycerol–Water Extraction of Selected Bioactive Compounds from Peppermint and Common Nettle. Antioxidants 2021, 10, 817. [CrossRef]

Huamán-Castilla, N.L.; Mariotti-Celis, M.S.; Martínez-Cifuentes, M.; Pérez-Correa, J.R. Glycerol as Alternative Co-Solvent for Water Extraction of Polyphenols from Carménère Pomace: Hot Pressurized Liquid Extraction and Computational Chemistry Calculations. Biomolecules 2020, 10, 474. [CrossRef] [PubMed]

Tsai, C.-E.; Lin, L.-H. DPPH scavenging capacity of extracts from Camellia seed dregs using polyol compounds as solvents. Heliyon 2019, 5, e02315. [CrossRef]

Jakupovic´, L.; Kalvarešin, M.; Bukovina, K.; Poljak, V.; Vujic´, L.; Koncˇic´, M.Z. Optimization of Two Eco-Friendly Extractions of Black Medick (Medicago lupulina L.) Phenols and Their Antioxidant, Cosmeceutical, α-Glucosidase and α-Amylase Inhibitory Properties. Molecules 2021, 26, 1610. [CrossRef] [PubMed]

Vieira, V.; Calhelha, R.C.; Barros, L.; Coutinho, J.A.P.; Ferreira, I.C.F.R.; Ferreira, O. Insights on the Extraction Performance of Alkanediols and Glycerol: Using Juglans regia L. Leaves as a Source of Bioactive Compounds. Molecules 2020, 25, 2497. [CrossRef]

Huaman-Castilla, N.L.; Martínez-Cifuentes, M.; Camilo, C.; Pedreschi, F.; Mariotti-Celis, M.; Pérez-Correa, J.R. The Impact of

Temperature and Ethanol Concentration on the Global Recovery of Specific Polyphenols in an Integrated HPLE/RP Process on Carménère Pomace Extracts. Molecules 2019, 24, 3145. [CrossRef]

Shi, J.; Yu, J.; Pohorly, J.; Young, J.C.; Bryan, M.; Wu, Y. Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. J. Food Agric. Environ. 2003, 1, 42–47.

Teh, S.-S.; Niven, B.E.; Bekhit, A.E.-D.; Carne, A.; Birch, E.J. The Use of Microwave and Pulsed Electric Field as a Pretreatment Step in Ultrasonic Extraction of Polyphenols from Defatted Hemp Seed Cake (Cannabis sativa) Using Response Surface Methodology. Food Bioprocess Technol. 2014, 7, 3064–3076. [CrossRef]

Liu, Z.; Esveld, E.; Vincken, J.-P.; Bruins, M.E. Pulsed Electric Field as an Alternative Pre-treatment for Drying to Enhance Polyphenol Extraction from Fresh Tea Leaves. Food Bioprocess Technol. 2019, 12, 183–192. [CrossRef]

Kokkali, M.; Martí-Quijal, F.J.; Taroncher, M.; Ruiz, M.-J.; Kousoulaki, K.; Barba, F.J. Improved Extraction Efficiency of Antioxidant Bioactive Compounds from Tetraselmis chuii and Phaedoactylum tricornutum Using Pulsed Electric Fields. Molecules 2020, 25, 3921. [CrossRef]

Saldaña, G.; Cebrián, G.; Abenoza, M.; Sánchez-Gimeno, C.; Álvarez, I.; Raso, J. Assessing the efficacy of PEF treatments for improving polyphenol extraction during red wine vinifications. Innov. Food Sci. Emerg. Technol. 2017, 39, 179–187. [CrossRef] [PubMed]

Pollini, L.; Cossignani, L.; Juan, C.; Mañes, J. Extraction of Phenolic Compounds from Fresh Apple Pomace by Different Non-Conventional Techniques. Molecules 2021, 26, 4272. [CrossRef] [PubMed]

Carpentieri, S.; Mazza, L.; Nutrizio, M.; Jambrak, A.R.; Ferrari, G.; Pataro, G. Pulsed electric fields- and ultrasound-assisted green extraction of valuable compounds from Origanum vulgare L. and Thymus serpyllum L. Int. J. Food Sci. Technol. 2021, 56, 4834–4842. [CrossRef]

Δημοσίευση VIII

Combination of Pulsed Electric Field and Ultrasound in the Extraction of Polyphenols and Volatile Compounds from Grape Stems.

George Ntourtoglou [1,2], Fotini Drosou [1], Theodoros Chatzimitakos [2] , Vassilis Athanasiadis [2] , Eleni Bozinou [2] ,Vassilis G. Dourtoglou [1] , Abeer Elhakem [3] , Rokayya Sami [4] , Amal Adnan Ashour [5] , Alaa Shafie [6] and Stavros I. Lalas [2],*

1. Department of Wine, Vine, and Beverage Sciences, School of Food Science, University of West Attica, 12243 Athens, Greece; gntourtoglou@uniwa.gr (G.N.); fdrosou@uniwa.gr (F.D.); vdourt@uniwa.gr (V.G.D.)
2. Department of Food Science and Nutrition, University of Thessaly, 43100 Karditsa, Greece;tchatzimitakos@uth.gr (T.C.); vaathanasiadis@uth.gr (V.A.); empozinou@uth.gr (E.B.)
3. Department of Biology, College of Science and Humanities in Al-Kharj, Prince Sattam Bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia; a.elhakem@psau.edu.sa
4. Department of Food Science and Nutrition, College of Sciences, Taif University, P.O. Box 11099,Taif 21944, Saudi Arabia; rokayya.d@tu.edu.sa
5. Department of Oral & Maxillofacial Surgery and Diagnostic Sciences, Faculty of Dentistry, Taif University,P.O. Box 11099, Taif 21944, Saudi Arabia; a.a.ashour@tu.edu.sa
6. Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University,P.O. Box 11099, Taif 21944, Saudi Arabia; a.shafie@tu.edu.sa

ABSTRACT

Increasing the yield of extraction of bioactive compounds from plants is of high importance. Grape stems are widely discarded during the wine making process, despite their high content in many valuable compounds. The aim of this work was to examine whether the use of pulsed electric field (PEF) treatment of the stems could increase the yield of polyphenol and volatile compounds in the extracts. For this reason, a relatively low energy consuming PEF process was employed (low electric field strength, 1 kV/cm) for a short time (30 min) at the grape stems. In addition, the effect of different solvents during this pretreatment step was examined. With the use of Folin–Ciocalteu assay, the extracts were compared with the respective control samples (not pretreated with PEF). Moreover, extracts were prepared to assess whether changes occur to the volatile profile of the extracts. The results were conclusive that not only PEF can increase the yield of polyphenols (up to 35% increase recorded), but also that the solvent used during PEF pretreatment can affect the process. Furthermore, a 234% increase in the total content of volatile compounds was

recorded, when PEF was used as a pretreatment step. Therefore, the combination of PEF and ultrasound-assisted extraction is highly promising to obtain grape stem extracts with a higher content of bioactive compounds.

Keywords: by-product; extraction; phenolics; pulsed electric field; ultrasonication; gas chromatography–mass spectrometry

INTRODUCTION

Today, more than ever, there is a growing interest in sustainability, including, but not limited to crop by-products. Consequently, adding value to biowaste is more important than ever [1]. Considering and treating biowaste as a source of bioactive compounds can result in significant benefits in terms of sustainability, as well as major benefits to the food and beverage industries [2]. Grape stems are widely known materials that are left over from grapes and wine-making. Every year, millions of tons of waste are produced from the cultivation of grapes with only a small percentage of them (<4%) remaining for the feeding of animals. Grape stems represent about 5% of the waste from the process of wine-making, and in general, the production of grapes [3]. Regarding the grape stems, researchers worldwide have successfully identified their composition [4,5]. They mainly consist of water [6], cellulose and hemicellulose, lignin, proteins, acids, sugars, as well as polyphenolic compounds, particularly those derived from red grapes. The possibility of creating value from the by-products of the abovementioned process has been studied by several researchers [7–10]. For instance, Quero et al. [11] studied the antiproliferative effect of grape stem extracts against three cancer cell lines. Similarly, Leal et al. [7] studied the antioxidant, antimicrobial, anti-inflammatory, and anti-aging properties of the grape stem extracts. Of all their components, phenolic compounds are of great importance owing to their antioxidant, antibacterial, and antifungal properties [12–15]. To this end, multiple extraction techniques have been employed to prepare grape stem extracts, including solid–liquid extraction [16], ultrasonication (US) [17], and pressurized liquid extraction [9]. However, to the best of our knowledge, the pulsed electric field has not been employed to date. The pulsed electric field (PEF) is a green extraction technique that has been used in recent years to optimize the extraction process of precious substances [18–20]. PEF is an extraction technique, based on electropermeabilization, aiming at the creation of pores in the cellular membranes, and thus, assist in the extraction of compounds. Owing to its inherent advantages, PEF has been successfully

employed for the improved extraction of polyphenols from citrus fruits [21], potato peels [22], Sideritis raiseri [23], Merlot grapes [24], etc. Moreover, it has been shown that the use of PEF in combination with ultrasound can increase the extraction of phenolics from plant by-products [25]. The PEF is typically applied prior to the ultrasound-assisted extraction as a pretreatment step. However, this step does not exclude the release of ingredients from the raw materials during PEF treatment. In particular, short-duration pulses between 100 ns–1 ms are used with a voltage between 1 and 3 kV.

The present study aimed at investigating whether the use of PEF prior to the ultrasound- based extraction of polyphenols from grape stems can enhance their extraction, and to what extent. In addition, volatile compounds contained in the extracts were examined, since they are valuable molecules widely used in the food industry. To this end, grape stem extracts were prepared, using the ultrasound treatment. Moreover, PEF pretreated samples (with various solvents) were subjected to the ultrasound treatment and compared.

MATERIALS AND METHODS

Chemicals

Folin–Ciocalteu reagent and all solvents used were at least of analytical grade and purchased from Chem Lab (Zedelgem, Belgium).

Plant Material and Sample Processing

Grape stems from the Merlot variety were obtained from Kavala, Greece region. The stems were collected in airtight bags and placed in a freezer during transportation. Prior to further processing, stems were washed with deionized water and dried using paper towels. Then, 30 g of stems were chopped into smaller pieces (between 45 and 50 mm). The stems were equally shared within 6 beakers (5.0 g in each beaker). Half of the beakers were used to prepare the control samples and the others for the PEF treated samples. Extraction was carried out in two steps. In the first step, grape stems were immersed in a solvent and subjected to PEF treatment. In the second step, the PEF treated and non-treated samples were subjected to ultrasonic extraction. More specifically, three sets of experiments were carried out: (I) In one beaker, the stems were left intact, without the addition of any medium. Simultaneously, the second portion of stems was inserted into the PEF treatment chamber. After 30 min, the stems were transferred into two Duran bottles (250 mL) and 50 mL of extraction solvent (methanol:deionized water, 70:30 v/v) was added to fully cover the stems. (II) For the second experiment, another 5 g of stems were inserted in a 250 mL Duran bottle with 50 mL of deionized water. At the same time, 50 mL of deionized water with 5 g of stems were added to the PEF treatment chamber. After 30 min (with or without the PEF treatment), the solvent was retracted and subjected to further analysis. The stems in

both cases were transferred to new containers and the extraction solvent (methanol:deionized water, 70:30 v/v) was added. (III) For the third experiment, 5 g of stems were inserted in a 250 mL Duran bottle and followed by the addition of a methanol:water mixture (50:50 v/v). The same mixture of solvents along with 5 g of stems was added to the PEF treatment chamber. Following the treatment, the solvent was retracted (and subjected to further analysis) and the stems were transferred to new beakers, where the extraction solvent (methanol:deionized water, 70:30 v/v) was added. In all cases, after the addition of the final extraction solvent, the mixtures (solvent and stems) were placed in an ultrasonic bath for 15 min. Each experiment was repeated three times.

Instrumentation

The equipment used for the PEF treatment is provided in detail in our previous studies [19,20,23]. In brief, the system was comprised of a high voltage power generator (Leybold, LD Didactic GmbH, Huerth, Germany), a digital oscilloscope, a function/arbitrary waveform generator, and a treatment chamber made of two stainless steel plates (10 cm length and 10 cm height) and 1 cm Teflon between them for isolation purposes. The pulse duration used for the treatment of the samples was 1 ms and the frequency of the pulses was 1 Hz. The wave type is a typical square wave with a maximum delay of 20 ns. Absorbance measurements were carried out at a Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany). Ultrasound treatment of the samples was carried out at a TRANSONIC 570/H (ELMA) unit, with a volume of 4.25 L, frequency of 35 KHz, and HF peak of 320 W.

GC–MS analyses were carried out using an Agilent 6890 series GC System (Agilent Technologies, Santa Clara, CA, USA), coupled to a 5975C MSD mass detector. A fused silica capillary column (30 m 0.32 mm i.d. 0.25 μm film thickness (HP-5MS, Agilent Technologies) was used for the separation of compounds, and helium was used as carrier gas at a flow rate of 1 mL/min. The sample (1 μL) was injected using a split ratio of 100:1. The injector temperature was set at 250 ◦C and the temperature of the transfer line was set at 280 ◦C. The detector was operated at the ionization mode (EI), using a voltage of 70 eV, and spectra were recorded in the mass range of 40–550 amu. Oven temperature was set at 70 ◦C for 0.5 min, increased to 100 ◦C with a rate of 8 ◦C per min and remained for 0.5 min, and finally, increased to 250 ◦C with a rate of 5 ◦C per min and remained for 15 min. The total run time was 49.75 min. Data were recorded with the Turbomass 5.0 ChemStation software. Identification of the compounds was carried out using the NIST 11 library.

Measurement of Total Phenolic Content

The measurement of total phenolic content (TPC) is based on the oxidation of phenols in an alkaline environment (Folin–Ciocalteu method) [26,27]. In a vial containing 7.9 mL of deionized water and 500 μL of Folin–Ciocalteu reagent, 100 μL of the samples (as mentioned in Section 2.2) were added. After mixing at a vortex for 1 min, 1.5 mL of saturated Na2CO3 solution was added. A blank sample was prepared by substituting the sample with deionized water. After mixing again in a vortex, samples were incubated at 40 ◦C for 30 min. Finally, absorbance spectra were recorded in the range of 500–800 nm and comparisons between samples were carried out by measuring the

absorbance at 765 nm. Results were expressed as absorbance units (AU) and not as equivalents of other compounds, with calibration curves.

Measurement of Volatile Compounds

Extracts of the control samples and the PEF treated samples (using methanol as solvent), after ultrasound extraction, were dried using sodium sulfate. After filtering, using syringe filters, 10 μL of internal standard solution (3-octanol in pentane) was added, and 1 μL of the sample was inserted into the GC–MS system.

Statistical Analysis

All of the experiments were performed in triplicates. Results are expressed as means of all measurements (three replicate extractions x three analyses = nine total measurements). Statistically significant differences were evaluated using ANOVA, followed by Duncan's multiple range test for $p < 0.05$. Statistical analysis was carried out using SPSS (SPSS Inc., Chicago, IL, USA) software.

RESULTS AND DISCUSSION

Phenolic Content of the Extracts

To date, a significant amount of effort has been placed to examine the phenolic content of extracts obtained solely by ultrasound treatment of plant mixtures and lesser effort to study the effect of PEF treatment. In addition, studies focusing on the combination of the two aforementioned techniques are insufficient and in short supply [25,28]. The results obtained from PEF pretreated and non-pretreated samples subjected to ultrasound extraction are summarized in Table 1. In accordance with the results, the use of PEF without the aid of ultrasonication does not lead to notable changes in the polyphenolic content of the extracts (the recorded increase in the TPC was <4% and was not found to be statistically significant). This minuscule increase in the TPC can possibly be attributed to the fact that even though the cell membranes are partially damaged, the phenolic compounds cannot, yet, be released [25]. On the contrary, when PEF was used as a pretreatment step, prior to the ultrasonication extraction step, a notable increase in the polyphenolics contained in the extract was recorded. More specifically, when 1:1 v/v methanol:water was used as a solvent, during the PEF pretreatment step, a 17% increase in the polyphenolic content of the extract (after US extraction) was recorded, compared with the non-pretreated sample. Similarly, when water was used as a solvent during the PEF pretreatment step, the respective extract contained 35% more polyphenols, compared with the non-pretreated sample with PEF extract.

Table 1. Absorption of samples, resulting from various treatments, using the Folin–Ciocalteu method. Statistically significant differences ($p < 0.05$) are denoted with superscript letters; AU: Absorbance units.

Solvent Used before Treatment	PEF Treatment	US Extraction	Extract from the Control Beaker (AU)	Extract from the PEF Beaker (AU)	% Increase in TPC
only stems	✗	✓	0.081 ± 0.001^{a}	0.084 ± 0.002^{a}	4
50% v/v methanol:water	✓	✗	0.053 ± 0.001^{a}	0.055 ± 0.001^{a}	4
	✓	✓	0.086 ± 0.006^{a}	0.101 ± 0.005^{b}	17
water	✓	✗	0.051 ± 0.001^{a}	0.053 ± 0.001^{a}	4
	✓	✓	0.085 ± 0.005^{a}	0.115 ± 0.005^{b}	35

In a previous study, a similar approach was employed to examine whether PEF pretreatment can increase the TPC content of rosemary and thyme extracts, obtained by ultrasound-assisted extraction [25]. In accordance with the authors, the use of PEF as a pretreatment step was found to be beneficial in both cases. However, the PEF pretreatment was found to increase the TPC of thyme extracts, compared with rosemary extracts. In another study, the PEF pretreatment increased by nearly 15% of the TPC content of almond extract (obtained by US-assisted extraction), compared with PEF non-pretreated extract [28]. Therefore, in accordance with our results, it can be concluded that not only PEF can be used as a pre-treatment step to maximize the extraction yield of polyphenols, but the solvent used during the PEF treatment step, is also of high importance to obtain the optimum extract.

Effect on Volatile Compounds

It is known that there are cases of wine-making in which stems are used to bestow the wine with green and vegetal aroma [6]. Therefore, grape stems can be used as a source to extract aroma compounds for further use in the food industry. In this context, we examined whether treating stems with PEF, prior to the US-assisted extraction of the volatile compounds, can also increase the yield of aroma compounds. Representative chromatograms of the extracts are provided in Figure 1 and the results in Table 2. In the samples extracted without PEF pretreatment, a total of six volatile compounds were identified, whose total content was 0.73 mg/Kg. Regarding the extracts obtained using PEF treatment, prior to the US-assisted extraction, a total of eight volatile compounds were identified, whose total content was 2.44 mg/Kg. The new compounds identified were 1,14-tetradecanediol and 1-methoxy-4-methyl-benzene. Therefore, PEF pretreatment increased the total volatile con- tent of the extract by 234%. There are even more pronounced increases for some individual compounds, such as 4-dodecanol, whose content was increased by 2100%, and hexanedioic acid, bis(2-ethylhexyl) ester, whose content increased by 716%. In all cases (except for 14- and 16-heptadecenal), the content of each compound was found to be statistically significantly higher in PEF pretreated samples. Therefore, our results highlight the superiority of PEF for use as a pretreatment step. The composition of volatile components of the grape stems is an understudied area, resulting in a lack of data regarding this topic. Hashizume et al. [29] examined the volatile components of grape stems of the Cabernet Sauvignon and

Chardonnay varieties and identified seven compounds. Similarly, Ruiz-Moreno et al. [30] identified seven compounds in the Syrah variety grape stems. Since grape stems can contribute unique aromas to the wines, further research should focus on the determination of volatile profiles of grape stems to obtain wines with the desired properties.

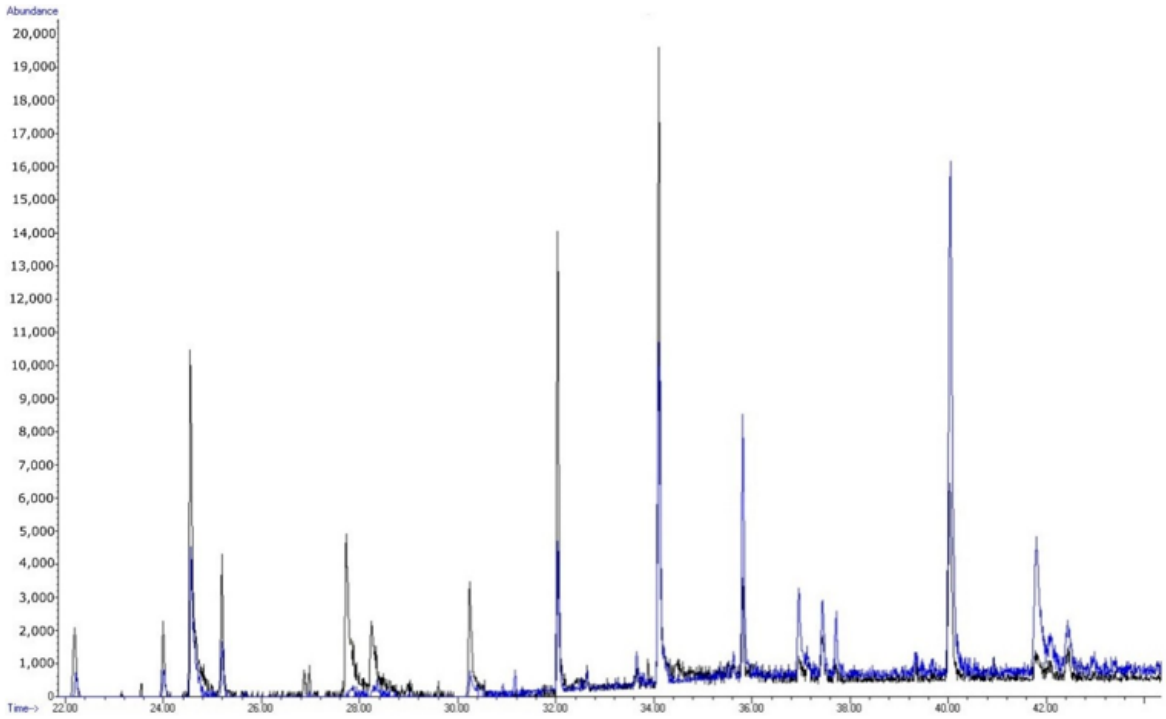


Figure 1. Representative chromatograms of grape stem extracts obtained by ultrasound-assisted extraction (blue chromatogram) and obtained by ultrasound-assisted extraction, accompanied by a PEF pretreatment step (black chromatogram).

Table 2. Volatile compounds detected by gas chromatography–mass spectrometry in PEF treated and non-treated samples. Statistically significant differences ($p < 0.05$) are denoted with superscript letters.

Compounds	Control (mg/Kg)	PEF (mg/Kg)	% Increase
Phenylethyl Alcohol	0.04 ± 0.01 ^{a}	0.10 ± 0.02 ^{b}	150
Benzene, 1-methoxy-4-methyl-	ND	0.07 ± 0.02	-
n-Hexadecanoic acid	0.10 ± 0.03 ^{a}	0.59 ± 0.09 ^{b}	490
1,14-Tetradecanediol	ND	0.39 ± 0.14	-
4-Dodecanol	0.01 ± 0.01 ^{a}	0.22 ± 0.01 ^{b}	2100
Hexanedioic acid, bis(2-ethylhexyl) ester	0.06 ± 0.01 ^{a}	0.49 ± 0.05 ^{b}	716
14-Heptadecenal	0.13 ± 0.02 ^{a}	0.15 ± 0.02 ^{a}	15
16-Heptadecenal	0.39 ± 0.09 ^{a}	0.43 ± 0.05 ^{a}	10
Total volatile content	*0.73*	*2.44*	*234*

CONCLUSIONS

Grape stems are a natural by-product of the wine-making industry, which contains valuable compounds. These compounds can be retrieved using various extraction techniques, with the most common as ultrasound-assisted extraction. In accordance with our results, the use of PEF as a pretreatment step can significantly enhance the extraction of polyphenolic compounds from grape stems. Moreover, it was apparent that the use of different solvents during PEF treatment of the stems can further increase the yield of polyphenols. Similarly, PEF not only was found to increase the yield of volatile compounds in the extracts, but also assisted in the extraction of two additional volatile compounds, compared with bare ultrasound-assisted extraction-derived extracts. As a concluding remark, the use of PEF as a pretreatment step in the extraction of various volatile substances as well as polyphenolic compounds, in a very short period (30 min) is a promising approach that can be utilized for the enhancement of the extraction process. Furthermore, by studying other parameters of the extraction, such as extraction solvents, time, and temperature, further enhancement of the extraction yield can be achieved.

Author Contributions: Conceptualization, S.I.L. and V.G.D.; methodology, T.C., G.N. and V.A.; software, T.C.; validation, V.A., T.C., G.N. and E.B.; formal analysis, G.N., F.D., T.C., V.A., E.B., A.E., R.S., A.A.A. and A.S.; investigation, G.N., F.D., V.A., E.B. and T.C.; resources, A.E., R.S., A.A.A. and A.S.; data curation, A.E., R.S., A.A.A. and A.S.; writing—original draft preparation, G.N. and F.D.; writing—review and editing, all authors; visualization, T.C.; supervision, S.I.L. and V.G.D.; project administration, S.I.L.; funding acquisition, A.E., R.S., A.A.A. and A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research has no extra funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Acknowledgments: Taif University Researchers Supporting Project Number (TURSP-2020/130), Taif University, Taif, Saudi Arabia.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Lange, L. Biorefineries: Increased value from biomass conversion. In Biomass Combustion Science, Technology and Engineering; Woodhead Publishing: Sawston, UK, 2013; pp. 278–295. ISBN 9780857091314.

Pascoalino, L.A.; Reis, F.S.; Prieto, M.A.; Barreira, J.C.M.; Ferreira, I.C.F.R.; Barros, L. Valorization of bio-residues from the processing of main portuguese fruit crops: From discarded waste to health promoting compounds. Molecules 2021, 26, 2624. [CrossRef] [PubMed]

Gouvinhas, I.; Queiroz, M.; Rodrigues, M.; Barros, A.I.R.N.A. Evaluation of the Phytochemistry and Biological Activity of Grape (Vitis vinifera L.) Stems: Toward a Sustainable Winery Industry. In Polyphenols in Plants; Elsevier: Amsterdam, The Netherlands, 2019; pp. 381–394.

Alonso, Á.M.; Guillén, D.A.; Barroso, C.G.; Puertas, B.; García, A. Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content. J. Agric. Food Chem. 2002, 50, 5832–5836. [CrossRef] [PubMed]

Anastasiadi, M.; Pratsinis, H.; Kletsas, D.; Skaltsounis, A.L.; Haroutounian, S.A. Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. LWT Food Sci. Technol. 2012, 48, 316–322. [CrossRef]

Blackford, M.; Comby, M.; Zeng, L.; Dienes-Nagy, Á.; Bourdin, G.; Lorenzini, F.; Bach, B. A review on stems composition and their impact on wine quality. Molecules 2021, 26, 1240. [CrossRef]

Leal, C.; Gouvinhas, I.; Santos, R.A.; Rosa, E.; Silva, A.M.; Saavedra, M.J.; Barros, A.I.R.N.A. Potential application of grape (Vitis vinifera L.) stem extracts in the cosmetic and pharmaceutical industries: Valorization of a by-product. Ind. Crops Prod. 2020, 154. [CrossRef]

Mora-Garrido, A.B.; Cejudo-Bastante, M.J.; Heredia, F.J.; Escudero-Gilete, M.L. Revalorization of residues from the industrial exhaustion of grape by-products. LWT 2022, 156, 113057. [CrossRef]

Nieto, J.A.; Santoyo, S.; Prodanov, M.; Reglero, G.; Jaime, L. Valorisation of grape stems as a source of phenolic antioxidants by using a sustainable extraction methodology. Foods 2020, 9, 604. [CrossRef]

Leal, C.; Santos, R.A.; Pinto, R.; Queiroz, M.; Rodrigues, M.; José Saavedra, M.; Barros, A.; Gouvinhas, I. Recovery of bioactive compounds from white grape (Vitis vinifera L.) stems as potential antimicrobial agents for human health. Saudi J. Biol. Sci. 2020, 27, 1009–1015. [CrossRef]

Quero, J.; Jiménez-Moreno, N.; Esparza, I.; Osada, J.; Cerrada, E.; Ancín-Azpilicueta, C.; Rodríguez-Yoldi, M.J. Grape stem extracts with potential anticancer and antioxidant properties. Antioxidants 2021, 10, 243. [CrossRef]

Alasalvar, C.; Grigor, J.M.; Zhang, D.; Quantick, P.C.; Shahidi, F. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. J. Agric. Food Chem. 2001, 49, 1410–1416. [CrossRef]

Acamovic, T.; Brooker, J.D. Biochemistry of plant secondary metabolites and their effects in animals. Proc. Nutr. Soc. 2005, 64, 403–412. [CrossRef] [PubMed]

Edreva, A.; Velikova, V.; Tsonev, T.; Dagnon, S.; Gürel, A.L.; Aktas, L. Stress-protective role of secondary metabolites: Diversity of functions and mechanisms. Gen. Appl. Plant Physiol. 2007, 34, 67–78.

Lin, D.; Xiao, M.; Zhao, J.; Li, Z.; Xing, B.; Li, X.; Kong, M.; Li, L.; Zhang, Q.; Liu, Y.; et al. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. Molecules 2016, 21, 1374. [CrossRef] [PubMed]

Jiménez-Moreno, N.; Volpe, F.; Moler, J.A.; Esparza, I.; Ancín-Azpilicueta, C. Impact of extraction conditions on the phenolic composition and antioxidant capacity of grape stem extracts. Antioxidants 2019, 8, 597. [CrossRef] [PubMed]

Sahpazidou, D.; Geromichalos, G.D.; Stagos, D.; Apostolou, A.; Haroutounian, S.A.; Tsatsakis, A.M.; Tzanakakis, G.N.; Hayes, A.W.; Kouretas, D. Anticarcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells. Toxicol. Lett. 2014, 230, 218–224. [CrossRef]

Lakka, A.; Bozinou, E.; Makris, D.P.; Lalas, S.I. Evaluation of pulsed electric field polyphenol extraction from Vitis vinifera, Sideritis scardica and Crocus sativus. ChemEngineering 2021, 5, 25. [CrossRef]

Pappas, V.M.; Lakka, A.; Palaiogiannis, D.; Athanasiadis, V.; Bozinou, E.; Ntourtoglou, G.; Makris, D.P.; Dourtoglou, V.G.; Lalas, S.I. Optimization of pulsed electric field as standalone “green” extraction procedure for the recovery of high value-added compounds from fresh olive leaves. Antioxidants 2021, 10, 1554. [CrossRef]

Ntourtoglou, G.V.; Drosou, F.; Enoch, Y.; Tsapou, E.A.; Bozinou, E.; Athanasiadis, V.; Chatzilazarou, A.; Dourtoglou, E.G.; Lalas, S.I.; Dourtoglou, V.G. Extraction of volatile aroma compounds from toasted oak wood using pulsed electric field. J. Food Process. Preserv. 2021, 45, e15577. [CrossRef]

El Kantar, S.; Boussetta, N.; Lebovka, N.; Foucart, F.; Rajha, H.N.; Maroun, R.G.; Louka, N.; Vorobiev, E. Pulsed electric field treatment of citrus fruits: Improvement of juice and polyphenols extraction. Innov. Food Sci. Emerg. Technol. 2018, 46, 153–161. [CrossRef]

Frontuto, D.; Carullo, D.; Harrison, S.M.; Brunton, N.P.; Ferrari, G.; Lyng, J.G.; Pataro, G. Optimization of Pulsed Electric Fields-Assisted Extraction of Polyphenols from Potato Peels Using Response Surface Methodology. Food Bioprocess Technol. 2019, 12, 1708–1720. [CrossRef]

Athanasiadis, V.; Pappas, V.M.; Palaiogiannis, D.; Chatzimitakos, T.; Bozinou, E.; Makris, D.P.; Lalas, S.I. Pulsed Electric Field- Based Extraction of Total Polyphenols from Sideritis raiseri Using Hydroethanolic Mixtures. Oxygen 2022, 2, 91–98. [CrossRef]

Delsart, C.; Ghidossi, R.; Poupot, C.; Cholet, C.; Grimi, N.; Vorobiev, E.; Milisic, V.; Peuchot, M.M. Enhanced Extraction of Phenolic Compounds from Merlot Grapes by Pulsed Electric Field Treatment. Am. J. Enol. Vitic. 2012, 63, 205–211. [CrossRef]

Tzima, K.; Brunton, N.P.; Lyng, J.G.; Frontuto, D.; Rai, D.K. The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products. Innov. Food Sci. Emerg. Technol. 2021, 69, 102644. [CrossRef]

Chanioti, S.; Tzia, C. Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. Innov. Food Sci. Emerg. Technol. 2018, 48, 228–239. [CrossRef]

Zhang, Y.C.; Schwartz, S.J. Analysis of Isoflavones in Soy Foods. In Handbook of Food Analytical Chemistry; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2005; Volume 2, pp. 519–535. ISBN 9780471709084.

Manzoor, M.F.; Zeng, X.A.; Rahaman, A.; Siddeeg, A.; Aadil, R.M.; Ahmed, Z.; Li, J.; Niu, D. Combined impact of pulsed electric field and ultrasound on bioactive compounds and FT-IR analysis of almond extract. J. Food Sci. Technol. 2019, 56, 2355–2364. [CrossRef] [PubMed]

Hashizume, K.; Samuta, T. Green Odorants of Grape Cluster Stem and Their Ability to Cause a Wine Stemmy Flavor. J. Agric. Food Chem. 1997, 45, 1333–1337. [CrossRef]

Ruiz-Moreno, M.J.; Raposo, R.; Cayuela, J.M.; Zafrilla, P.; Piñeiro, Z.; Moreno-Rojas, J.M.; Mulero, J.; Puertas, B.; Giron, F.; Guerrero, R.F.; et al. Valorization of grape stems. Ind. Crops Prod. 2015, 63, 152–157. [CrossRef]

Δημοσίευση IX

Pulsed electric field: A “green” extraction technology for biomolecular products from glycerol with fermentation of non-Saccharomyces yeasts

Evangelia A. Tsapou [1], George Ntourtoglou[1], Fotini Drosou[1], Panagiotis Tataridis [1], Stavros Lalas [2] and Vassilis Dourtoglou[1]*

1 Department of Wine, Vine and Beverage Sciences, School of Food Science, University of West Attica, Athens, Greece,
2 Department of Food Science and Nutrition, University of Thessaly, Karditsa, Greece

ABSTRACT

Glycerol is the main organic by-product of the biodiesel industry and it can be a source of carbon for fermentations or a substrate for biotransformations. This work investigates a process that uses pulsed electric field (PEF) to enhance polyol and propanediols extraction from a glycerol/glucose fermentation broth. Three different commercial, non-*Saccharomyces* strains, *Torulaspora delbrueckii* Prelude (Hansen), *Torulaspora delbrueckii* Biodiva 291 (Lallemand) and *Metschnikowia pulcherrima* (Lallemand) were studied. The results revealed that PEF had a positive impact on the extraction of polyols ranging from 12 to 191%, independently of fermentation conditions. *Torulaspora delbrueckii* Biodiva 291 (Lallemand) was found to be more efficient at pH 7.1. An optimized chromatography-based method for the qualitative and quantitative determination of the formed products evaluated. The experiments were carried out either in flasks or in a bioreactor.

Keywords: pulsed electric field, non-saccharomyces yeasts, mannitol xylitol, propanediols, glycerol

INTRODUCTION

Pulsed electric field (PEF) is a technique that has been used for extractions due to its capability to cause disorganization of plant or microbial cells by disrupting membranes and releasing cell metabolites from the inner to outer part of the cell (Tsapou et al., 2020). The disruption of the cell membrane due to electroporation is caused by high intensity PEF and leads to complex phenomena ranging from cell restructuring to cell death (Delsart et al., 2012; Ntourtoglou et al., 2020). Electropermeabilization of cells must be irreversible to cause inactivation of microorganisms. Electroporation was previously used as nonthermal treatment of liquid foods to inactivate microorganisms (Alvarez et al., 2003; Ntourtoglou et al., 2020; Tsapou et al., 2020). Biofuels represent a category of fuels derived from biomass and include among others ethanol, biodiesel, green diesel and biogas. Biodiesel is considered to be a great substitute for conventional petro-diesel, which is a fast- depleting fossil fuel and for this reason is gaining worldwide popularity (Yang et al., 2012; Monteiro et al., 2018). During the transesterification process of the biodiesel production, approximately 10% of crude glycerol is formed as a by-product. Utilizing the glycerol waste is of outmost importance not only due to its potential to be used for the production of value added products but also due to the high cost and environmental impact that is associated with its disposal. It is known that a general ratio between biodiesel production and the amount of generated residual glycerol indicates that for every 10 parts of biodiesel, one part glycerol is produced (Mitrea et al., 2017). It is very important to convert crude glycerol into valuable byproducts to control and improve the economic sustainability of biodiesel production. Crude glycerol usually contains various impurities, such as water, methanol, soap, fatty acids, and fatty acid methyl esters. (Luo et al., 2016). Traditionally, crude glycerol was refined to pure glycerol, a useful raw material for industries, such as foods and beverages, pharmaceuticals, cosmetics, tobacco, and textiles (Luo et al., 2016; Samudrala, 2019). Different pathways, such as those involved in oxidation, acetalization, esterification, dehydration, and others used in the refining process yielding high added value products, can be obtained from crude glycerol (Aroua and Cognet, 2020).

Recent studies have focused on the use of crude glycerol as carbon source in microbial fermentations (Sadhukhan et al., 2016; Wischral et al., 2016; Mitrea et al., 2017). Many yeast, bacterial, and fungal strains are capable of growing on glycerol used as carbon source because this substrate can be both oxidatively and reductively metabolized through dehydrogenases or dehydratases (Mitrea et al., 2017). Some of the metabolic compounds that can be obtained via microbial fermentation of glycerol are acetic, lactic, propionic, citric, succinic, and oxalic acids, butanol, propanediols (1,3- and 1,2-propanediol), polyols (mannitol, xylitol, arabitol), ethanol, dihydroxyacetone, single-cell oil, biomass, and polyunsaturated fatty acids among others (Luo et al., 2016; Mitrea et al., 2017). Some of the microorganisms that have already been studied in aerobic and anaerobic fermentations with crude and pure glycerol as substrate are *Clostridium*

beijerinckii, *Escherichia coli, Lactobacillus rhamnosus, Enterococcus faecalis Yarrowia lipolytica,* and *Lactobacillus brevis* (Murakami et al., 2016; Sadhukhan et al., 2016; Vivek et al., 2016; Wischral et al., 2016; Prabhu et al., 2020). In the wine and beer industry selected non-*Saccharomyces* yeasts were observed to produce glycerol in addition to ethanol and volatiles during alcoholic fermentation (Drosou et al., 2022). However, most of works focused on ethanol production and sugar consumption and some of them on biogenesis of volatiles. Glycerol and polyols were analyzed either using enzyme assays or HPLC.

To increase the amount of the final products an electrotechnique (PEF) was used in the post-fermentation extraction. High intensity of the fields induced by PEF have the effect of cell membrane disruption. This disturbance of the architectural structure of the membrane leading to a cell lysis for example or the fusion of protoplasts (Delsart et al., 2012; Tsapou et al., 2020). This phenomenon has resulted in pore formation in cell membranes and intercellular metabolites diffuse into the extracellular medium, due to a critical value of transmembrane potential, around 0.8–1V (Zimmermann, 1986). The aim of this work is the application of PEF in biotransformations by improving the extraction of certain bioactive compounds. Another aim is to investigate whether the results are associated with the efficiency of the fermentation process which is strongly influenced by various factors, including microbial characteristics (strain- microrganism, medium type and composition), as well as process parameters (e.g. power, frequency, electric field strength), treatment time, pH and temperature. Analytical techniques are also investigated. To conclude, studies under different operating conditions were made to verify the potential impact of PEF on the extraction of bioactive metabolites with the aim of optimizing the process to provide the optimum results for each application.

MATERIALS AND METHODS

Chemicals

Pyridine (Pyr), Acetic anhydride (AcOAc), diethyl ether (95%), anhydrous sodium sulfate, toluene, and hydrochloric acid (HCL), were purchased from Chem Lab (Athens, Greece). All reagents were of analytical quality. All the chemicals used for the preparation of the medium, were purchased from Sigma Aldrich (St. Louis, MO, United States).

Fermentation

- **Yeast strains**

Three non-Saccharomyces yeast strains were used for the fermentation: 1. Torulaspora delbrueckii Prelude (Hansen) (Prelude), 2. Torulaspora delbrueckii Biodiva 291 (Lallemand) (Biodiva), and 3. Metschnikowia pulcherrima (Lallemand) (Mets).

- **Medium A**

For the preparation of the synthetic medium for the main cultures, the following ingredients were dissolved in 1 L of deionized water: 1 $gl^{-}1$ of KH2PO4, 1 $gl^{-}1$ of K2HPO4, 2 $gl^{-}1$ of (NH4)2SO4, 0.2 $gl^{-}1$ of MgSO4.7H2O, 0.2 $gl^{-}1$ of ZnSO4.5H2O and yeast extract 1 $gl^{-}1$. The medium was prepared the day of the fermentation 80 ml of the abovementioned medium was prepared and transferred to 150 ml flasks which were autoclaved at 121°C for 15 min. The cultures were initially inoculated with 1 ml of a 100 h pre-culture of the required strain (the concentration of the cells was calculated and adjusted at approximately 10 $*10^{6}$ cells/ml). Cell counting was performed by microscopy, using a CX60 microscope (Olympus Corporation, Center Valley, United States) and a Thomas type hemocytometer. Viability was evaluated by the methylene blue method, according to Lange et al. (1993) and Drosou et al. (2021). Optical density (O.D.) for Biodiva and Prelude, were measured every day from zero time until the eighth day of the fermentation at 600 nm with a UV-Vis spectrophotometer (UV- 1700 PharmaSpec, Shimadzu).

TABLE 1 Results from the Blank and the fermentation in which a PEF treatment was held before the extraction. The results presented in gL^{-1}.

	pH = 4.3		Difference	pH = 7.1		Difference
Compound	Blank	PEF		Blank	PEF	
1,2-Propanediol, diacetate	0.04	0.11	+175%	0.11	0.32	+191%
1,3- Propanediol, diacetate	0.72	1.40	+94%	0.68	1.30	+91%
1,2,3,4,5-Penta-O-acetyl-D-xylitol	0.20	0.56	+180%	1.20	1.90	+58%
D-Mannitol hexaacetate	0.17	0.19	+12%	0.69	0.93	+35%
Triacetin	3.89	6.21	+60%	5.11	6.32	+24%

Culture conditions for flask experiments

Fermentations were performed in 150 ml fermentation flasks which contained 80 ml of synthetic medium A supplemented with 62.64 $gl^{-}1$ pure glycerol and 20 $gl^{-}1$ glucose. Three different fermentation flasks were used each one containing one of the following microorganisms. A) Torulaspora delbrueckii (Prelude), B) Torulaspora delbrueckii Biodiva 291 (Biodiva) and C) Metschnikowia pulcherrima (Mets). The fermentations were performed under constant stirring (750 rpm) and at 25°C. From the second day till the fifth day 1 ml of pure glycerol was further added in each flask every 24 h. All fermentations were performed in duplicate. The weight of each fermentation flask was recorded daily; and the weight loss noted corresponded to CO_2 formation during the

fermentation process. Samples were taken every 4 days to determine polyols. Polyols were analyzed at the end of fermentation (day 12 in all cases). pH settings was between 4.3 and 7.1.

Culture conditions for fermentor experiments

For the needs of these experiments, a custom-made bioreactor was constructed. The bioreactor is comprised of three main parts.

Part 1: The first part was the main container of the bioreactor with a total volume of 2 L.

Part 2: On top of the main container mounted the lid of the bioreactor its outputs and inputs (Figure 1), which is airtight when closed. The lid consists of six ports, three on each side. All ports could be opened or closed at any given time.

- The two middle ports (3, 4) are connected and separated from the rest of the system by a stainless-steel pipe.

- Within the liquid inlet, another stainless steel pipe is installed, as well as a separate sieve assisting the separation in the sift of solid particles.

- To avoid the exposure of the thermometer to the reaction media, the thermometer placed inside a stainless steel tube, containing a thermal conductive media (glycerol, water, etc.)

Part 3: the third part was the stirrer also made out of stainless-steel (as exhibited on Figure 1) which was positioned inside the main hole. The stirrer had two Rushton Type 5 impeller tubes, one on each side, with a 5-cm diameter each. The motor can reach a maximum rotation speed of 200 rpm.

The total assembly in Figure 1 consisted of the bioreactor, an air pump, the PEF equipment with the treatment chamber as described by Ntourtoglou et al. (2020), one collector for the samples, and two peristaltic pumps that could push the fluid from the bioreactor to the PEF treatment chamber and consequently from the chamber to the sample collector. In order to evaluate the bioreactor system, 1 L of the synthetic medium A enriched with 100 $gIL^{-}1$ glucose was prepared. The Torulaspora Biodiva was initially pre cultured in the same medium and then 1 ml of this preculture was used to inoculate the final medium in the bioreactor (concentration of the inoculum approximately at 10 *106 cells/mL). The inoculum was added aseptically to the bioreactor. Pure glycerol was added at a rate of 5 ml/24 h from the third day onwards (Bioreactor Fermentation). The pH adjusted either at 4.3 or at 7.1 in two series of distinctive experiments.

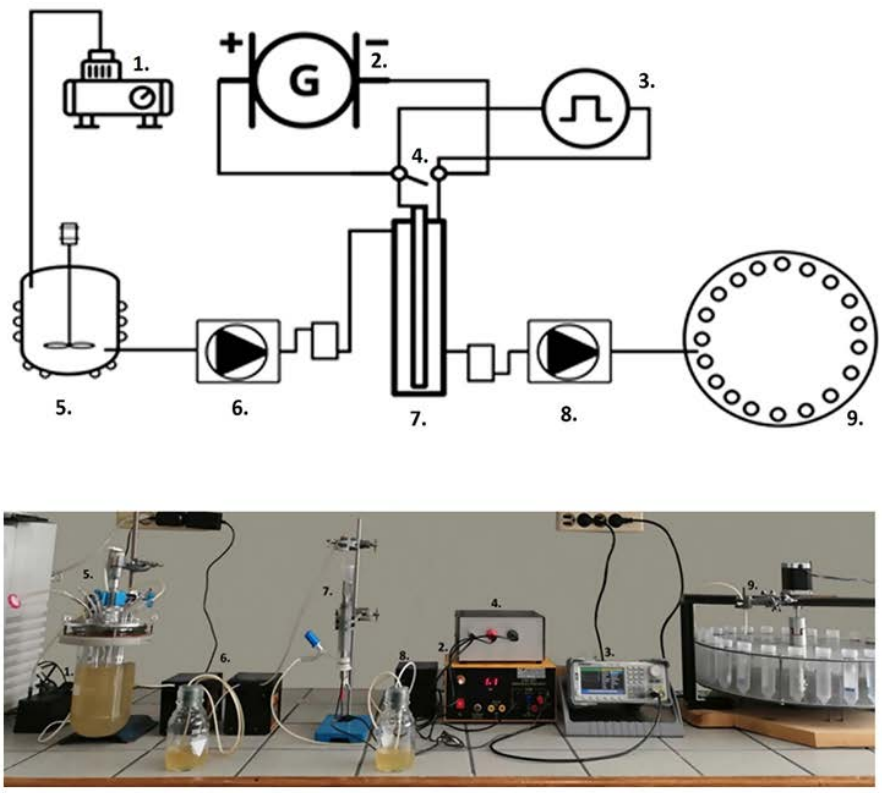


FIGURE 1
Process unit.

Nominal Volume, V (L)	2
Working volume, VL (L)	1.5
Impeller speed, TS (m/s)	0.5
Agitation speed, N (rmp)	60.00
Number of impeller	1
Impeller type	Rushton
Medium height, MH (cm)	10.5
Impeller diameter, DI (cm)	8
Reactor diameter, DT (cm)	20
Reactor hight, HT (cm)	10
MH/DT	2.12
DI/DT	0.30
Inoculum volume, Vx (0.1%)	0.001

PEF equipment

The PEF equipment consisted of a high voltage generator (eisco, eshrrr 1,338 model), an IGBT control that is used as a high frequency switch and a pulse generator (siglent,

SDG 1032X model) that controls the IGBT. The maximum voltage for the IGBT is 1100 V so the experiments took place with 1000 V for safety reasons. A square pulse with pick time 1 ms was created with a frequency of 1 Hz. The total treatment time was 30 min. The treatment chamber was a glass tube with 10 cm height and a diameter of 3 cm. Inside the tube, peripherally, there was perforated laminate stainless steel that took the shape of the exterior cylinder and in the middle there was a stainless steel cylinder with a diameter of 1 cm. The distance between the stainless steel cylinder and the perforated laminate stainless steel was 1 cm. The positive charge was in contact with the eccentric cylinder while the negative was joined with the peripherally perforated laminate stainless steel cylinder.

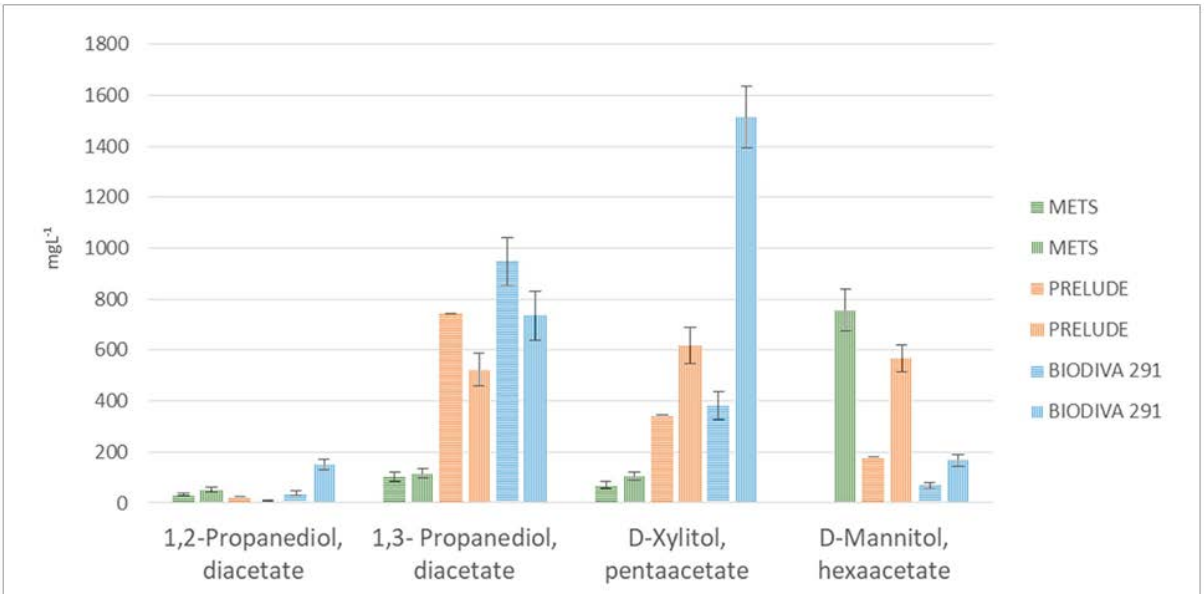


FIGURE 2
Results of the first series of flask fermentation (evaluation of yeast strains). Means of compounds in mgL^{-1} and standard deviations (n = 2).

Bioreactor combined with PEF extraction

For the fermentation and PEF extraction, the synthetic medium was enriched with 62.64 $gl^{-}1$ pure glycerol and 20 $gl^{-}1$ glucose. Pure glycerol was added at a rate of 1 ml/24 h for 4 days. Two bioreactors with 1 L medium and cells, as working volume, were cultured batch-wise for each pH value, one as the blank and the other consisted of a PEF treatment performed before the extraction of fermentation products.

Sample preparation for GC/MS

20 ml of each sample was centrifuged twice at 18.33 m/s for 10 min to separate the phases (Hermle Z200A, Milan, Italy). Five milliliters of the supernatants were condensed in a flash evaporator (IKA RV 10) until a final concentration of 100 mg. Then the samples were mixed with Pyr and AcOAc at concentrations of 1,000 μl each, and left for 24 h in 63°C. After that, samples were extracted twice with 20 ml of diethyl ether and washed with 2 ml of 5% HCL and 1 ml of deionized water in a separation funnel. The organic layer was washed twice with 5 ml of distilled water dried over anhydrous sodium sulfate and filtered. Two milliliters of toluene were added twice to remove traces of Pyr and condensed until a concentration of ≈150 mg was achieved. Finally, 100 μl of dichloromethane was added to the samples from which 1.0 μL was used for GC/MS analysis. The results of all batches were presented in gL-1 and calculated by a standard triacetin curve.

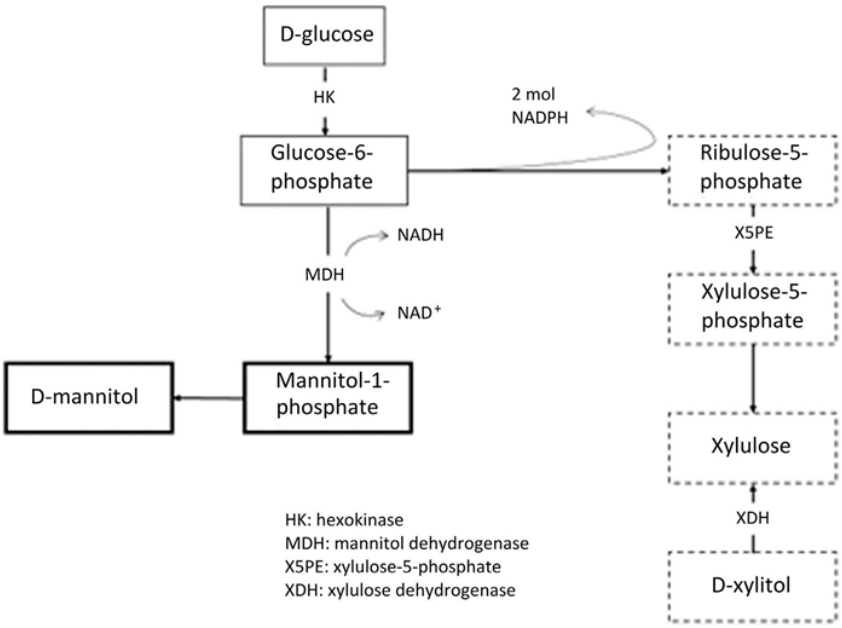


FIGURE 3
The pentose phosphate pathway and other metabolic routes for polyol production with glucose carbon source.

Gas chromatography/mass spectrometry analysis

The apparatus was an Agilent 6,890 series GC System (Agilent Technologies, Santa Clara, United States), equipped with 5975C VLMSD and an Agilent MG-HT-1 fused silica capillary column that was 30 m × 0.32 mm interior diameter (i.d.) × 0.25 μm film thickness. Moreover, samples were injected in a split ratio of 50:1. The injector temperature was set at 180°C, the carrier gas was helium at a flow rate of 1 ml/min, and the oven temperature was set initially at 50°C and was increased to 200°C at a rate of 10°C/min and maintained for 5 min. The temperature of the transfer line was set at

280°C. The mass spectrometer was operated in the ionization mode (EI) at an ionization voltage of 70 eV over masses ranging from 40 to 550 amu and a manifold temperature of 270°C. Data were recorded with Turbomass 5.0 ChemStation software (Agilent).

Statistical analysis

Standard deviations were carried out with Excel 2013 (Microsoft, Redmond,WA, United States).

RESULTS AND DISCUSSION

One of the objectives of this study was to optimize chromatography-based methods for polyol identification and quantification. After derivatization, using acetic acid anhydride, to synthetize polyols esters and Gas Chromatography coupled with Mass Spectrophotometry (GC-MS) seemed to be a powerful tool for polyol analyses in fermentation samples with or without residual sugars.

To evaluate the PEF technique as a downstream process in the extraction of products, more accurate product identification and analysis was necessary. From one point of view Gas chromatography/mass spectrometry (GC/MS) is an analytical technique, which gives accurate identification of the results. The conversion of products into their acetate esters provided an opportunity to be analyzed in more detail using with GC/MS (Table 1). From another point of view polyol esters are easily dissolved in organic solvents, thus, the extraction method used for the acetate esters combined with the PEF technique as a rapid “green” extraction technology was evaluated through the steps of microbial fermentation. Three strains of non-Saccharomyces yeasts were used to for the conversion of pure glycerol in biodiesel production.

Fermentation in flasks for optimal pH determination

From the first batch of fermentations, it was observed that all three strains were capable of producing propanediols and polyols during fermentation with glycerol (Figure 2). Concerning the efficacy of the fermentation at different pH levels, higher results for all strains were obtained in neutral pH. Mannitol and Xylitol are the polyols produced directly from glucose via its isomerization to fructose and they need the NADH/$NADH_2$ cofactor. As a result, a short aeration period is needed at the beginning of fermentation to avoid the displacement of NADH/ $NADH_2$ equilibrium. In a previous work of Mbuyane et al.(2018), three strains of Torulaspora delbrueckii were used to

produce polyols in a synthetic grape juice-like medium containing 120 gl⁻1 sugars. The results demonstrated that all strains were capable of producing polyols, such as d-arabitol, d-mannitol, d-xylitol, d-sorbitol, and ribitol. Previously Onishi and Suzuki (1968) stated that high concentrations of nitrogen sources and KH2PO4 in the medium remarkably decreased mannitol yield in spite of good utilization of the substrate using Torulaspora. Mannitojuciens. The produced mannitol was in yield of 31% consumed at optimal condition. They also stated that using washed yeast cells for the fermentation process gave much higher mannitol yield. Except for the carbon source, oxygen availability also studied during polyols production yield. Khan et al. (2009) investigated the production of mannitol from glycerol by resting cells of Candida magnolia and showed that oxygen availability influences positively the conversion, while potassium phosphate and excessive quantity of resting cells has a negative result. Lee et al. (2003), mentioned that Candida magnoliae produced mannitol using a mixture of fructose and glucose, as carbon source, with a yield of 83%. They specifically achieved a production of 213 gL-1 mannitol in the fed- batch fermentation of C. magnoliae using a glucose:fructose mixture at a ratio of 1:20. C. magnoliae is also reported to produce glycerol (Sahoo and Agarwal, 2002), erythritol (Koh et al., 2003) and xylitol (Tada et al., 2004), using different substrates and at different fermentation conditions.

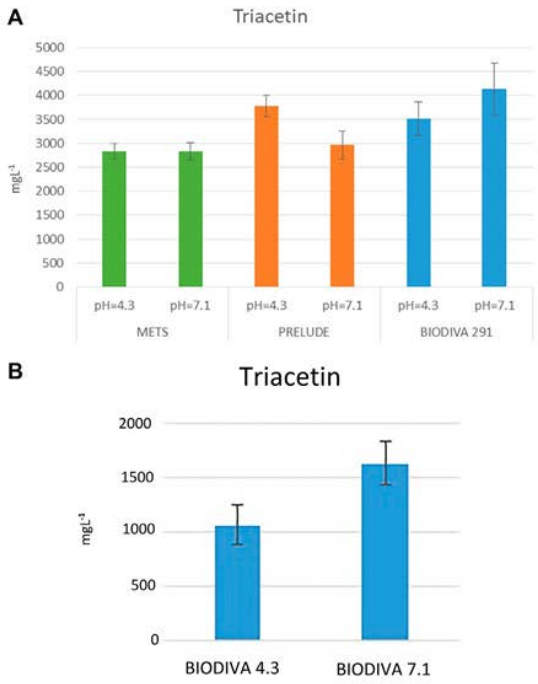


FIGURE 4
Evaluation of concumption of glycerin. Results of triacetin of (A) flask fermentation and (B) bioreactor fermentation, at pH 4.3 and 7.1. Means of triacetin in mgl^{-1} and standard deviations (n = 2).

In the present study, xylitol was produced from all examined strains under both acidic and neutral conditions, while mannitol was detected only at neutral pH for Mets and Prelude fermentation and at both pH for Biodiva.

As the carbon source is not just glycerol but also a mixture of glycerol and glucose, it is normal to find all polyols present either from the glucose-fructose pathway (Figure 3) or from glycerol (propanediols). In addition, the influence of pH cannot be predicted and is derived from each experiment with different osmophilic yeasts.

The initial concentration of glycerol for all fermentations for this series of experiments was 62.64 $gl^{-}1$. Comparing the concentrations of triacetin at the end of fermentation (Figure 4A), showed that the three strains were capable of consuming it as an energy source. Mets had the highest consumption while showing the lowest concentrations of 1,3-propanediol, mannitol, and xylitol. In Biodiva fermentation, higher amounts of the same products were analyzed, while having the lowest consumption efficiency of triacetin at 93% compared with the two other species (Figure 2). In Prelude fermentation, equal amounts of both glucose and triacetin were detected, a fact that might indicate that this strain needs more than 1 week of fermentation to yield the corresponding results.

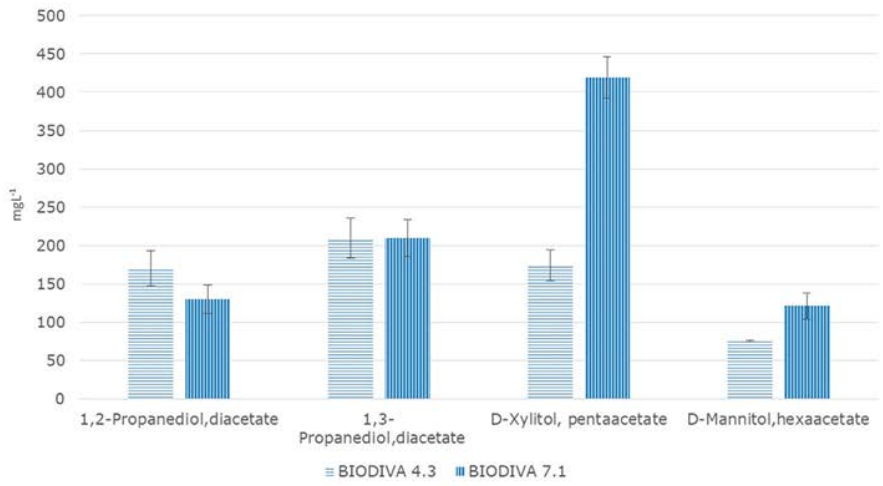


FIGURE 5
Results of bioreactor fermentation (evaluation of yeast strain in various pH). Means of compounds in mgL^{-1} and standard deviations (n = 2).

Mannitol and xylitol were quantified at higher concentrations in Biodiva samples. Specifically, for acidic pH, xylitol was found to be approximately five times higher in Prelude and Biodiva when compared with Mets. On the other hand, in a neutral pH, these two polyols were found at levels approximately fifteen times higher than in Biodiva and six times higher than in Prelude when compared with Mets.

Concerning mannitol in an acidic pH, was found at a concentration of 0.18 $gl^{-}1$ only after Biodiva fermentation. In a neutral pH, all strains were found to have mannitol, with the highest concentration (0.76 $gl^{-}1$) in Biodiva samples (Figure 2).

Both propanediols, 1,2- and 1,3-propanediol, were analyzed from all three strains at both pH values. 1,3- Propanediol had a higher concentration than 1,2- propanediol in all fermentation products. In Mets fermentation, the concentrations did not show much difference according to pH. In contrast, for Prelude and Biodiva, higher amounts of 1,3-propanediol were found at neutral pH. The higher concentrations found in Biodiva were 0.95 and 0.74 $gl^{-}1$ at pH values of 4.3 and 7.1, respectively, for 1,3-propanediol and for 1,2-propanediol quantified in 0.15 and 0.04 $gl^{-}1$ for pH 4.3 and 7.1, respectively. In the eighth day of fermentations (end time point), dry weight was measured for Biodiva and Mets and it was found to be 400 and 328 mg respectively. According to the results, Biodiva modified the medium into valuable products in higher amounts during a 1-week fermentation
period.

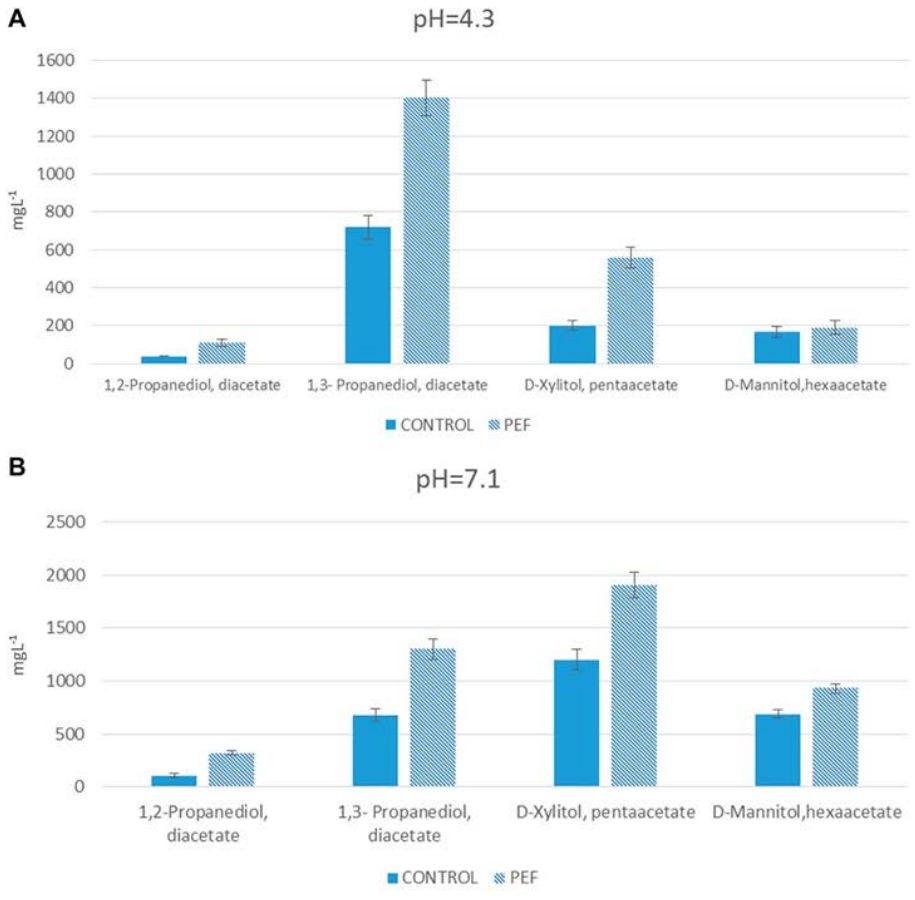


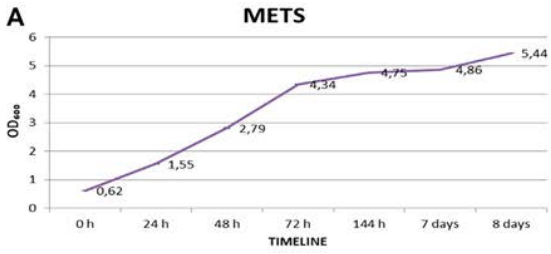
FIGURE 6
Results of bioreactor fermentation and PEF assisted extraction of polyols, at pH (A) 4.3 and (B) 7.1. Means of compounds in mgL^{-1} and standard deviations (n = 2).

Fermentation in bioreactor

In order to scale up the fermentation, validate the pH of medium composition, and the source of the carbon, bioreactor cultivations were carried out in a 2.00 L scale bench bioreactor with 1 L working volume. The process conditions were identical as in the shake flask studiy except for the aeration. The aeration rate was maintained at 2.0 L/min for the first 72 h and then stopped. Two separate batch fermentations were ran, with the Biodiva strain. One at pH 4.3 and using glucose/glycerol as the carbon sources and the other at pH 7.1 with the same carbon substrates. The time course profiles for both fermentations were similar to that of the shake flask cultivations. Glycose is the most preferred carbon source for Biodiva and in the presence of glycerol the uptake of xylose was repressed as evident in Figure 2 and Figures 5, 6. NADP/H2 availability is in most cases a rate-limiting factor in the reduction of xylose to xylitol. There are three ways that microbial cells produce polyols.

The first is a direct hydroxyl elimination leading to purivate derivatives propane diols, the second is an isomerization of glycose to maltitol and other polyols and the last one is from ribulose 5-phosphate, as shown in Figure 2. In the work of Tavares et al. (1999) it is shown for the first time that xylitol production by the yeast Debaryomyces hansenii is not only a result of a redox balance usually occurred under poor aerobic conditions, but also that there are additional physiological mechanisms involved, mainly from phosphate

limitation.



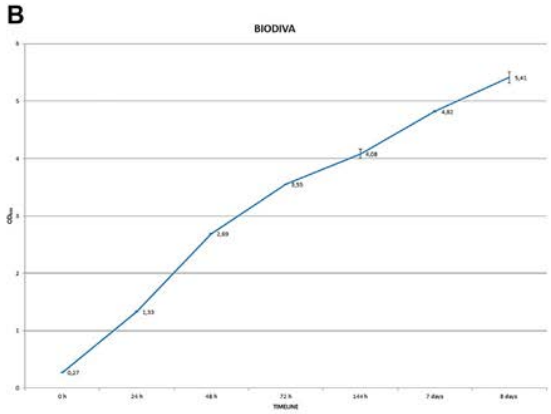


FIGURE 7
Time course profiles of (A) *Metschnikowia pulcherrima* and (B) *Torulaspora delbrueckii* on OD_{600}, for flask cultivation. Means and standard deviations (n = 2).

In the next series of experiments, the bioreactor was used for the Biodiva fermentation process in the glucose-glycerol medium to allow the first step for the scale up of the production polyols from laboratory bench to industrial levels (Figure 4). In this batch, the medium contained 100 $gl^{-}1$ of glucose and 23.2 $gl^{-}1$ of glycerol. From Figures 4,7, it was concluded that the consumption efficiency of glycerol was 93% for neutral pH and 95% for the acidic pH. The concentration of triacetin in addition to the initial concentration of glycerol was lower when compared with the results from the shake flask studies, indicating that the consumption of glycerol at acidic pH was higher, but the amounts of polyols were lower when compared with neutral pH. Better results were obtained again at neutral pH.

Specifically, for propanediols, their concentrations were almost the same with no significant difference at either pH values. As a sum, their concentrations were lower with the flask fermentation (for Biodiva), 0.38 $gl^{-}1$ and 0.39 $gl^{-}1$ for acidic and neutral pH values, respectively, while in flask fermentation, the concentrations were 0.99 $gl^{-}1$ and 0.89 $gl^{-}1$, respectively. In polyol production, an increase in this batch was noted, which was lower for mannitol and higher for xylitol, and at neutral pH yielded better results for both polyols. As a sum of the concentration of these two polyols and after comparing flask and bioreactor fermentation, an increase in the results from bioreactor

fermentations was found. Particularly, in acidic pH, the increase was 42.8%, while in neutral pH the increase was 20.4%.

Application of PEF for increase the extractability of polyols

For the last experiment, a third batch was examined. At both neutral and acidic pH levels, the concentration of valuable compounds in the PEF samples were higher. Most of the metabolites are intracellular, such as polyols (Kasumi, 1995), and this is the main reason of the evaluation of PEF, to enhance polyol extraction from a glycerol/glucose fermentation broth. The most important difference was found between the blank and PEF samples with percentages ranging between 191 and 12%. Specifically, the percentage of mannitol in neutral pH was improved by 35% after the PEF treatment and in acidic pH by 12%. Finally, from the initial amount of glycerol that was added at the start of the experiment, an average of 91% was consumed with the highest value in acidic pH in the blank and lowest in neutral pH in the blank. The highest concentration of triacetin in both PEF samples was normal because the yeast cells produce glycerol to maintain a stable osmotic level. It is well known that during PEF treatment, the cell membrane expands and the products that are trapped inside the membrane escape into the environment (Angersbach et al., 2000).

CONCLUSION

In conclusion, it was demonstrated that the use of PEF could increase the yield of the fermentation products of three non-Saccharomyces yeast strains using glycerol/glycose as a carbon source. The experiment was performed both at small volumes (flasks) and at bioreactors (as an up-scaling process). Between the three non-Saccharomyces yeasts, Biodiva 291 (Lallemand) (Biodiva) was the strain which seemed to be more efficient in the biotransformation process. According to our findings, the PEF-treated samples showed higher concentrations of compounds in both acidic and neutral environments. The PEF treatment was shown to enhance the concentration of compounds after fermentation from 12 to 180% in an acidic environment and from 24 to 191% in a neutral environment. Specifically xylitol has an increase of 180% in acidic pH and 1,2- propanediol has an increase of 175 and 191% in acidic and neutral pH respectively. From our results, we can reach the conclusion that the PEF treatment aids and enhances the isolation of the fermentation products. Therefore should be suggested that this technique has the potential to be used for industrial applications and should be further investigated.

Data availability statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions: ET: Formal analysis, analysis of volatile compounds and Writing—Original Draft GN: Pulsed electric Field and Bioreactor design and construction, Writing—Original Draft FD: Fermentations. PT: Microorganisms culture. SL: Writing—Review & Editing. VD: Project administration, Supervision, Writing—Review & Editing.

Funding: This research was funded by the project entitled “Adding value to biodiesel-derived crude glycerol with the use of Chemical and Microbial Technology” (Acronym: Addvalue2glycerol; grant number T1EΔK-03002), which is financially supported by the Ministry of National Education and Religious Affairs, Greece (project action “Investigate—Create—Innovate 2014–2020, Intervention II”).

Acknowledgments: We are thankful to Dermitzaki, Emmanouela and Mikroulis, Theodore, for the verification of our results from Vioryl SA.

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

REFERENCES

Alvarez, I., Pagan, R., Condon, S., and Raso, J. (2003). The influence of process parameters for the inactivation of Listeria monocytogenes by pulsed electric fields. Int. J. Food Microbiol. 87 (1–2), 87–95. doi:10.1016/S0168-1605(03)00056-4

Angersbach, A., Heinz, V., and Knorr, D. (2000). Effects of pulsed electric fields on cell membranes in real food systems. Innov. Food Sci. Emerg. Technol. 1, 135–149. doi:10.1016/S1466-8564(00)00010-2

Aroua, M. K., and Cognet, P. (2020). Editorial: From glycerol to value-added products. Front. Chem. 8, 69. doi:10.3389/fchem.2020.00069

Delsart, C., Ghidossi, R., Poupot, C., Cholet, C., Grimi, N., Vorobiev, E., et al. (2012). Enhanced extraction of phenolic compounds from merlot grapes by pulsed electric field treatment. Am. J. Enology Vitic. 63, 205–211. doi:10.5344/ajev.2012. 11088

Drosou, F., Anastasakou, K., Tataridis, P., Dourtoglou, V., and Oreopoulou, V. (2022). Evaluation of commercial strains of Torulaspora delbrueckii in beer production. J. Am. Soc. Brew. Chem. 80 (3), 1–10. doi:10.1080/03610470.2021. 2025327

Drosou, F., Anastasakou, K., Tataridis, P., Dourtoglou, V., and Oreopoulou, V. (2021). Study of the fermentation kinetics and secondary metabolites of Torulaspora delbrueckii yeasts from different substrates. J. Am. Soc. Brew. Chem. 1, 99–108. doi:10.1080/03610470.2021.1915660

Kasumi, T. (1995). Fermentative production of polyols and utilization for food and other products in Japan. JARQ 29, 49–55.

Khan, A., Bhide, A., and Gadre, R. (2009). Mannitol production from glycerol by resting cells of Candida magnoliae. Bioresour. Technol. 100 (20), 4911–4913. doi:10. 1016/j.biortech.2009.04.048

Koh, E.-S., Lee, T.-H., Lee, D.-Y., Kim, H.-J., Ryu, Y.-W., and Seo, J.-H.(2003). Scale up of erythritol production by an osmophilic mutant of Candida magnoliae. Biotechnol. Lett. 25, 2103–2105. doi:10.1023/b.bile. 0000007076.64338.ce

Lange, H., Bavouzet, J. M., Taillandier, P., and Delorme, C. (1993). Systematic error and comparison of four methods for assessing the viability of Saccharomyces cerevisiae suspensions. Biotechnol. Tech. 7, 223–228. doi:10. 1007/bf02566152

Lee, J.-K., Song, J.-Y., and Kim, S.-Y. (2003). Controlling substrate concentration in fed batch Candida magnoliae culture increases mannitol production. Biotechnol. Prog. 19, 768–775. doi:10.1021/bp034025

Luo, X., Ge, X., Cui, S., and Li, Y. (2016). Value-added processing of crude glycerol into chemicals and polymers. Bioresour. Technol. 215, 144–154. doi:10. 1016/j.biortech.2016.03.042

Mbuyane, L. L., Kock, M., Bauer, F. F., and Divol, B. (2018). Torulaspora delbrueckii produces high levels of C5 and C6 polyols during wine fermentations. FEMS Yeast Res. 18, 7. doi:10.1093/femsyr/foy084

Mitrea, L., Trif, M., Cătoi, A. F., and Vodnar, D. C. (2017). Utilization of biodiesel derived-glycerol for 1, 3-PD and citric acid production. Microb. Cell Fact. 16, 190. doi:10.1186/s12934-017-0807-5

Monteiro, M. R., Kugelmeier, C. L., Pinheiro, R. S., Batalha, M. O., and da Silva Cesar, A. (2018). Glycerol from biodiesel production: Technological paths for sustainability. Renew. Sustain. Energy Rev. 88, 109–122. doi:10.1016/j.rser.2018. 02.019

Murakami, N., Oba, M., Iwamoto, M., Tashiro, Y., Noguchi, T., Bonkohara, K., et al. (2016). L-Lactic acid production from glycerol coupled with acetic acid metabolism by Enterococcus faecalis without carbon loss. J. Biosci. Bioeng. 121 (1), 89–95. doi:10.1016/j.jbiosc.2015.05.009

Ntourtoglou, G., Tsapou, E. A., Drosou, F., Bozinou, E., Lalas, S., Tataridis, P., et al. (2020). Pulsed electric field extraction of α and β-acids from pellets of humulus lupulus (hop). Front. Bioeng. Biotechnol. 8, 297. doi:10.3389/fbioe.2020.00297

Onishi, H., and Suzuki, T. (1968). Production of D-mannitol and glycerol by yeasts. Appl. Microbiol. 16 (12), 1847–1852. doi:10.1128/am.16.12.1847-1852.1968, No

Prabhu, A. A., Thomas, D. J., Ladesma- Amaro, R., Leeke, G. A., Medina, A., Verheecke- Vaessen, C., et al. (2020). Biovalorisation of crude glycerol and xylose into xylitol by oleaginous yeast Yarrowia lipolytica. Microb. Cell Fact. 19, 121. doi:10.1186/s12934-020-01378-1

Sadhukhan, S., Villa, R., and Sarkar, U. (2016). Microbial production of succinic acid using crude and purified glycerol from a Crotalaria juncea based biorefinery. Biotechnol. Rep. 10, 84–93. doi:10.1016/j.btre.2016. 03.008

Sahoo, D. K., and Agarwal, G. P. (2002). Effect of oxygen transfer on glycerol biosynthesis by an osmophilic yeast Candida magnoliae I2B. Biotechnol. Bioeng. 78, 545–555. doi:10.1002/bit.10237

Samudrala, S. P. (2019). “Glycerol transformation to value-added 1, 3- propanediol production: A paradigm for a sustainable biorefinery process,” in Glycerine Prod. Transform. Innov. Platf. sustain. Biorefinery Energy, IntechOpen. Chap. 5, 24. doi:10.5772/intechopen.83694

Tada, K., Horiuchi, J.-I., Kanno, T., and Kobayashi, M. (2004). Microbial xylitol production from corn cobs using Candida magnoliae. J. Biosci. Bioeng. 98, 228–230. doi:10.1016/S1389-1723(04)00273-7

Tavares, J. M., Duarte, L. C., Amaral-Collaco, M., and Girio, F. M. (1999). Phosphate limitation stress induces xylitol overproduction byDebaryomyces hansenii. FEMS Microbiol. Lett. 171 (2), 115–120. doi:10.1111/j.1574-6968.1999.tb13420.x

Tsapou, E. A., Ntourtoglou, G., Drosou, F., Tataridis, P., Dourtoglou, T., Lalas, S., et al. (2020). In situ creation of the natural phenolic aromas of beer: A pulsed electricfield applied to wort-enriched flax seeds. Front. Bioeng. Biotechnol. 8, 583617. doi:10.3389/fbioe.2020.583617

Vivek, N., Pandey, A., and Binod, P. (2016). Biological valorization of pure and crude glycerol into 1, 3-propanediol using a novel isolate Lactobacillus brevis N1E9.3.3. Bioresour. Technol. 213, 222–230. doi:10.1016/j.biortech. 2016.02.020

Wischral, D., Zhang, J., Cheng, C., Lin, M., De Souza, L. M. G., Pellegrini Pessoa, F. L., et al. (2016). Production of 1, 3-propanediol by Clostridium beijerinckii DSM 791 from crude glycerol and corn steep liquor: Process optimization and metabolic engineering. Bioresour. Technol. 216, 100–110. doi:10.1016/j.biortech.2016.04.020

Yang, F., Hanna, M., and Sun, R. (2012). Value-added uses for crude glycerol–a byproduct of biodiesel production. Biotechnol. Biofuels 5, 13. doi:10.1186/1754-6834-5-13

Zimmermann, U. (1986). Electrical breakdown, electropermeabilization andelectrofusion.Rev. Physiol. Biochem. Pharmacol. 105, 175–256. doi:10.1007/bfb0034499